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KALLE KOIVUNIEMI

BIOELECTRICITY PRODUCTION FROM SIMULATED MINING AND FOREST INDUSTRY WASTEWATERS IN MICROBIAL FUEL CELLS

Master of Science Thesis

Examiners: D.Sc. Aino-Maija Lakaniemi and M.Sc. Mira Sulonen
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ABSTRACT

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The globe is facing various challenges arising from the increase of population and change of consumer habits. Electricity and pure water are essential for modern societies, and technologies to ensure their availability must be developed. Microbial fuel cells (MFCs) are devices which can be used to generate electricity from wastes and wastewaters using microbial metabolism.

In this study two different application fields of MFCs were experimented: 1) the degradation of tetrathionate to yield electricity and 2) the optimal MFC start-up strategies when using forest industry wastewaters as substrate. The capabilities of extreme acidophiles *Acidithiobacillus ferrooxidans* and *Ferroplasma acidiphilum* to degrade tetrathionate and generate electricity from simulated mining industry wastewater were studied in aerobic batch cultivations and in anaerobic two-chamber MFCs. Tetrathionate was readily degraded in aerobic cultivations by *A. ferrooxidans*, whereas no degradation by *F. acidiphilum* was observed. Tetrathionate was not degraded and electricity generation was minimal in MFCs inoculated with one or both of the cultures, most likely due to insufficient mixing and low biomass in the MFCs.

Different MFC start-up strategies were evaluated with air-cathode MFCs using simulated forest industry wastewater as substrate. Four strategies were compared: gradually decreasing external resistance from 5000 Ω to 50 Ω on weekly basis, stable low external resistance (50 Ω), controlled high anode potential (0 mV vs. Ag/AgCl), and controlled low anode potential (-450 mV vs. Ag/AgCl). Controlled high anode potential started MFC produced the highest maximal and average current densities during the start-up (38 days), 72 A/m^3 and 14 A/m^3, respectively. Controlled low anode potential was the least successful method with 0.17 A/m^3 average current density during the start-up. Chemical oxygen demand increased during the start-up, likely due to solubilisation of organic matter from the inoculum, but started to decrease in all MFCs as they were subjected to 47 Ω external resistance.

This study suggests that *A. ferrooxidans* was the observed tetrathionate degrading microorganism but its role as an electrogen in the MFC experiments needs further verification. Controlled high anode potential was found the most recommendable MFC start-up method, considering the current and power production, when using forest industry wastewater as the substrate.

TIIVISTELMÄ

KALLE KOIVUNIEMI: Biologinen sähköntuotanto simuloiduista kaivos- ja metsäteollisuuden jätevesistä mikrobipolttokennoissa
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Avainsanat: mikrobipolttokenno, MFC, puhdasviljelmä, tetrationaatti, jätevesi, *Acidithiobacillus ferrooxidans*, *Ferroplasma acidiphilum*, käynnistys, metsäteollisuuden jätevesi

Kulutustottumusten muutokset sekä väkiluvun kasvu aiheuttavat uusia haasteita, joihin ihmiskunnan on vastattava. Sähkö ja puhdas vesi ovat elintärkeitä hyödykkeitä nyky-yhteiskunnalle, joten teknologioita jotka turvaavat niiden saatavuuden tulee edelleen kehittää. Mikrobipolttokennot (MFC) ovat laitteita, jotka voivat tuottaa sähköä jätteistä ja jätevesistä mikrobien aineenvaihdunnan avulla.

Tässä diplomityössä tutkittiin kahta eri MFC:jen sovellusaluetta: 1) tetrationaatin hajottamista sähköä tuottaen sekä 2) optimaalista MFC:n käynnistysstrategiaa käytettäessä metsäteollisuuden jätevettä substraattina. Kahden äärimmäisen happamissa oloissa elävän mikrobin, *Acidithiobacillus ferrooxidans* ja *Ferroplasma acidiphilum*, kykyjä hajottaa tetrationaattia simuloidusta kaivosjätevedestä tutkittiin aerobisissa panospullokasvatuksissa sekä anaerobisissa kaksikammioisissa MFC:issa. Tetrationaatin havaittiin kuluvan aerobisissa kasvatuksissa *A. ferrooxidansin*, muttei *F. acidiphilumin* toimesta. Tetrationaatti ei hajonnut ja sähköntuotanto oli minimaalista yhtä sekä molempia organismeja sisältäneissä MFC:issa, todennäköisesti riittämättömän sekoituksen ja vähäisen biomassan määrän vuoksi.

MFC:jen eri käynnistysmenetelmiä arvioitiin ilmakatodilla varustetuilla MFC:illa käyttäen simuloitua metsäteollisuuden jätevettä substraattina. Neljää eri meneltemää vertailtiin keskenään: ulkoisen resistanssin laskemista asteittain 5000 Ω:sta 50 Ω:iin, pysyvää matalaa ulkoista resistanssia (50 Ω), korkeaksi säädettyä anodipotentiaalia (0 mV vs. Ag/AgCl) sekä matalaksi säädettyä anodipotentiaalia (-450 mV vs. Ag/AgCl). Korkeaksi säädetyn anodipotentiaalin MFC tuotti korkeimman maksimaalisen sekä keskimääräisen virrantiheyden (72 A/m^3 ja 14 A/m^3) käynnistysvaiheen (38 vrk) aikana. Matalaksi säädetty anodipotentiaali todettiin vähiten onnistuneeksi menetelmäksi 0,17 A/m^3 keskimääräisellä virrantiheydellä. Kemiallinen hapenkulutus nousi käynnistysvaiheen aikana, todennäköisesti siirrosteen mukana tulleen orgaanisen aineksen hajoamisen vuoksi, mutta alkoi laskea kaikissa MFC:issa kun ne asetettiin 47 Ω ulkoiseen resistanssiin.

Toteutetun tutkimuksen perusteella *A. ferrooxidans* oli varsinainen tetrationaattia hajottanut mikro-organismi, mutta sen roolia sähköntuottajana ei voitu todentaa MFC-kokeissa. Korkeaksi säädetty anodipotentiaali todettiin tehon ja virrantuoton kannalta suositeltavimmaksi MFC:n käynnistysmenetelmäksi käytettäessä metsäteollisuuden jätevettä substraattina.

PREFACE

This Master of Science Thesis was conducted in the Department of Chemistry and Bioengineering of Tampere University of Technology as part of BioElectroMET and Bio-e-MAT projects. The funding, provided by Europe's Seventh Framework Programme (FP7/2012-2016; Grant agreement no. 282970) and The Academy of Finland (New Indigo ERA-Net Energy 2014; Project no. 283013), is highly appreciated.

As my thesis project is drawing to an end, I want to begin my free word with thanks to my instructors: Aino-Maija for belief, flexibility and first-class commenting, and Mira for good company and all the superb MFC wizardry. Also thanks to mum and dad who are always super interested, even though the technical details may be a notch too much. Special thanks to my ever so disturbed lunch company who have had to bear all the whining and the mediocre puns. And the last thanks of course to Ida, who supported me all the way, tolerated the long days in the lab and tried to get the work out of my mind.

Tampere, 28 July 2016

Kalle Koivuniemi

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TERMS AND DEFINITIONS

A. ferrooxidans	*Acidithiobacillus ferrooxidans*
AMD	Acid mine drainage
BOD	Biochemical oxygen demand
COD	Chemical oxygen demand
COD_s	Soluble COD. Measured from filtered sample
COD_{tot}	Total COD. Measured from unfiltered sample
DoxDA	Thiosulphate quinone oxidoreductase
DSMSA	Disulphane monosulphonic acid
emf	Electromotive force
F. acidarmanus	*Ferroplasma acidarmanus*
F. acidiphilum	*Ferroplasma acidiphilum*
GDR	Gradually decreasing external resistance MFC start-up strategy.
HAP	High anode potential MFC start-up strategy.
HPLC	High performance liquid chromatography
LAP	Low anode potential MFC start-up strategy.
LSV	Linear sweeping voltammetry
MES	Microbial electrochemical system
MFC	Microbial fuel cell
MSM	Mineral salts medium
NER	Normalised energy recovery
NHE	Normalised hydrogen electrode
OCV	Open circuit voltage
OD	Optical density
R_{ext}	External resistance
R_{int}	Internal resistance
RISC	Reduced inorganic sulphur compound
SCE	Saturated calomel electrode
SLR	Stable low external resistance MFC start-up strategy
TES	Trace element solution
TetH	Tetrathionate hydrolase protein
VFA	Volatile fatty acid

ε_{Cb}	Coulombic efficiency
F	Faraday constant, 96 485 C/mol
ΔG_r	Gibbs free energy
ΔG_f^0	Gibbs free energy in standard conditions
I	Current
M	Molecular mass
P	Power
P_v	Power density against the anode chamber volume
Q	Flow rate
T	Temperature
t	Time
U	Voltage
V	Volume

1. INTRODUCTION

In the early days of the third millennium, the humanity is facing major challenges caused by industrialisation and the subsequent increases of world population and utilisation of natural resources. As the planet Earth is rapidly warming due to anthropogenic action (IPCC 2014), there is a real need for technologies that allow us to harness electricity using less adverse natural resources and to ensure the availability of pure water for everyone. Furthermore, mankind is increasingly dependent on natural resources mined or pumped from the Earth's crust to be consumed as energy, smartphones, fertilisers, and many other products that define the lifestyle in 2010's.

It has been estimated that 5–20% of all used water is utilised by the industry (Corcoran et al. 2010). The wide use of water reflects also the amount of wastewater produced by the industry. Industrial wastewaters can be divided into two major classes: diffuse industrial pollutants and end-of-pipe point discharges (UN-Water 2015). Industrial wastewaters not only end up to pollute rivers and lakes, but may also seep into the ground and contaminate groundwater. According to Corcoran *et al*. (2010), modern industrial processes utilise a wide array of complex organic molecules and heavy metals which are a potential risk to human and environmental health. Some of the industries that generate the largest volumes of toxic industrial waste are mining, pulp mills, tanneries, sugar refineries, and pharmaceutical production (Corcoran et al. 2010).

For more than a century, the activated sludge process has been the standard wastewater treatment procedure used all over the world in various domestic and industrial plants. Although activated sludge process is a valid option for purification of certain waters containing organic contaminants, it is energy intensive due to the need of extensive aeration (Wei et al. 2003). New technologies to valorise and exploit wastes are in the centre of the attention, as they not only allow more efficient recirculation of natural resources and reduction of the amount of waste, but also offer new business models (O'Callaghan 2016). Thus, in the emerging circular economy, one's waste will be a valuable resource for somebody else.

Microbial fuel cells (MFCs) are a technology which has the capability of becoming a major form of bioenergy in the future (Pant et al. 2010). MFC is a microbial electrochemical system (MES) that uses microorganisms to oxidise organic or inorganic matter and donate the resulting electrons to an electrode, thus generating electric current (Logan et al. 2006; Pant et al. 2010). The MFCs consist of anode and cathode electrodes, linked with electric wires through which the electrons produced at the anode are transferred to the cathode where they are used to reduce the terminal electron acceptor. Anodic and

cathodic chambers are typically linked via a membrane which allows the excess protons or other produced metabolites to transfer from the anode chamber to the cathode chamber or the other way around. To make a MFC, the system must retain living cells that directly influence the function of the MES (Logan et al. 2006). Pure or mixed cultures of microorganisms are traditionally used in the anode compartment to degrade compounds and provide electrons to the system, but they may also be beneficial at the cathode compartment e.g. regenerating terminal electron acceptors.

MFCs are a promising technology to treat wastewaters (Logan et al. 2006; Pant et al. 2010). The systems that use mixed microbial consortia are typically robust to changes in their environment (Rittman & McCarty 2001) which makes MFCs a potential technology in treating various different wastewaters and environmental pollutants. MFCs can be used to recover metals from wastewaters (ter Heijne et al. 2010), produce electricity to run wastewater treatment plant (Pant et al. 2010) or decrease the chemical oxygen demand (COD) of the water (Venkata Mohan et al. 2008). Forest industry wastewaters characteristically contain fermentable sugars (Pokhrel & Viraraghavan 2004) that are commonly used substrates for MFCs. Mining industry wastewaters characteristically contain metals and other compounds in concentrations that are toxic to most microorganisms, limiting the use of biological wastewater treatment (Dopson & Holmes 2014a). However, these wastewaters can be utilised as substrate in some extremophilic MFCs (Dopson & Holmes 2014a).

The aim of this thesis was to provide efficient ways to recover chemical energy stored in certain industrial wastewaters, to find efficient ways to degrade environmental pollutant tetrathionate, and to study the optimal MFC start-up strategies when using simulated forest industry wastewater as the substrate for anode microorganisms. In the first part of the study, I examine the capabilities of pure cultures of two extreme acidophiles, *Acidithiobacillus ferrooxidans* and *Ferroplasma acidiphilum*, to degrade tetrathionate from simulated mining wastewater in MFCs. Aerobic tetrathionate degradation potential of the pure cultures was studied in batch bottle experiments, and the potentiality of the cultures to produce electricity from tetrathionate was studied in two-chamber MFCs, with ferric iron as the terminal electron acceptor. Air-cathode MFCs were used to degrade simulated forest industry wastewater utilising four different MFC start-up strategies: applying gradually decreasing external resistance, low external resistance, controlled high anode potential, and controlled low anode potential.

In Chapter 2, I introduce the reader to wastewaters and their treatment as well as MFCs by literature survey on the subject. In Chapter 3, I present how the methods of the study and in Chapter 4 the results found in the study. I discuss the results in scientific context in Chapter 5, and in the last chapter I conclude what was achieved in the study.

2. THEORETICAL BACKGROUND

In this chapter, I examine the theoretical background of the thesis in relation to the literary information. The chapter begins with an examination of common industrial wastewaters and their characteristics in Section 2.1. After that, in Section 2.2, I describe how wastewaters are treated biologically. I study MFCs in more detail in Section 2.3, and how wastewaters can be utilised as MFC substrates in Section 2.3.4. In the last section (2.4) I describe how biotechnology can be used to harness extremophilic life in MFCs.

2.1 Industrial wastewater characteristics

Industrial wastewaters are remarkably different from domestic wastewaters. Madigan *et al*. (2009) state that domestic wastewater encompasses mainly gray water, sewage and food processing wastewater, whereas industrial wastewaters may contain high amounts of toxic compounds such as toxic chemicals, heavy metals and toxic organic compounds. The characteristics of industrial wastewaters are typically highly variable, as they are affected by various process related operations such as the manufactured product, operation start-ups, and shut-downs (Munter 2003).

Industrial water consumption is high, approximately 5–20% of all used water, even though closed water cycles have become more frequent in recent years in all areas of the industry, especially in extremely water-consuming cooling water systems (Corcoran et al. 2010). The largest three industrial users of fresh water globally are, according to Gupta and Bhardwaj (2016), metal industry, chemical industry and forest industry. The extensive use of fresh water reflects also to large amount of wastewater, which must be treated before its runoff back to the nature (Gupta & Bhardwaj 2016). According to Munter (2003), the industries produce wastewater roughly as much as municipal sources if electric power industry, which uses large amounts of cooling water, is excluded.

2.1.1 Mining industry

Mining industry generates significant amounts of waste annually. In 2012, mining and quarrying generated 29.2% of all waste produced in Europe and was surpassed only by construction industry (Eurostat 2016). According to Lottermoser (2010), all mine waters contain major concentrations of cations and anions, but the compositions of the mine waters show great variation depending on the geographical location and extracted metals or minerals. Sivakumar *et al*. (1992) claim that the mining wastewaters contain typically physical (suspended solids, colour and odour), organic (soaps, oils, dyes an phenols), inorganic (heavy metals, acids, alkalis, cyanide, salts), biological (bacteria, viruses) as well as radiological (uranium) contaminants. They exhibit typically high total dissolved solids

and hardness but low BOD, COD and suspended solids (Dharmappa et al. 2009). According to Lottermoser (2010), it is important to notice that the mine water releases do not necessarily result in damage to the environment. Even elevated metal concentrations do not cause harm if the elements are not readily bioavailable or if they are not taken up by plants and/or animals (Lottermoser 2010). However, Dopson *et al*. (2014b) state that all metals are toxic in high enough concentrations.

In metallic sulphide mining habitats, acid mine drainage (AMD) may occur. In AMD process the spontaneous oxidation of sulphide minerals in the presence of air and water acidifies the water. The exposure may be caused by mining activities but it can also happen naturally, in which case it is called acid rock drainage (Lottermoser 2010). Acidification of the environment makes it hostile to all but acidophilic organisms, and many metals are more soluble in acidic pH (Dopson et al. 2014b) leading to elevated metal concentrations in AMD waters.

2.1.2 Forest industry

The industry that uses wood as the main raw material is called forest industry. It can be roughly divided to wood product industry, such as sawmill industry and carpentry, and chemical forest industry, such as pulp and paper industry. Pulp and paper industry is one of the largest industries in the world and it has been steadily growing in the recent years (Gupta & Bhardwaj 2016). However, according to Finnish Forest Industry (Salo 2015) the growth of the global demand has stabilised in recent years to approximately 400 million tonnes of paper products per annum.

Lignocellulose is the part of woody biomass which consists of carbohydrates cellulose and hemicellulose tightly bound to aromatic lignin. Lignin provides rigidity to the wood cell wall with its complex aromatic structure (Mishra & Thakur 2016). It is not constructed from repeating subunits, which makes its enzymatic hydrolysis extremely difficult. Cellulose provides a skeleton to which lignin and hemicellulose are bound to (Mishra & Thakur 2016). Hard wood contains large amounts of lignin compared to soft wood (Gupta & Bhardwaj 2016). Pulping process itself generates highly concentrated wastewater and thus the biochemical oxygen demand (BOD) of pulping wastewater is directly linked to used soft wood / hard wood ratio. (Gupta & Bhardwaj 2016).

The substance spectrum in forest industry wastewater is wide. According to Munter (2003), pulp and paper mill wastewaters contain typically multiple different heavy metals: Cr, Cu, Hg, Pb, Ni, and Zn. Other chemicals that may have adverse effects on health or environment, e.g. mercaptans and sulphides, are also present. The waters are characterised by high BOD and chemical oxygen demand (COD), colour, organochlorines, and total dissolved and suspended solids. They contain high concentrations of dissolved organic compounds and fibrous suspended solids which are in a dissolved form. Small organic molecules cause the wastewater BOD, and lignin and its derivatives attribute to the

raise in COD. Pulp bleaching is responsible for most of the toxicity in the wastewater. The effluent from the paper industry is characterised also by organic halogen compounds and high concentrations of nitrogen and phosphorous, whereas wastewaters from chemical pulping encompass an array of toxins such as chlorine and its derivatives. (Gupta & Bhardwaj 2016).

The recent boom in biofuel production has evoked the interest in biofuel production from woody biomass. Thus, it has been also in the centre of attention of traditional chemical forest industry companies who boast wide knowhow from biorefining. There are already companies that are commercially producing biofuels from woody biomass such as UPM (renewable diesel) from pulping process side streams (UPM 2016) and Blue Marble Biomaterials (biogas and chemical components) from agricultural and forestry by-products (Blue Marble Biomaterials 2016).

2.2 Biological wastewater treatment

Wastewaters are typically remediated with chemical, physical or biological methods. The methods are not exclusive and usually the most effective treatment is achieved by combination of several methods (Madigan et al. 2009). As an example, phosphorous removal by chemical precipitation is a typical technique used in conjunction with biological methods (Rittman & McCarty 2001). Anaerobic digestion is used in conjunction with many wastewater treatment operations as it allows the extraction of energy from e.g. biomass derived from the sludge produced in aerobic wastewater treatment processes.

Activated sludge process is a typical way to decrease the BOD of the wastewater. The key of the activated sludge process is flocculation which allows the separation of the microbial biomass from the effluent in a settling tank and their reintroduction back to the process. The flocs are formed by electrostatic forces and naturally produced organic polymers (Madigan et al. 2009). According to Rittman and McCarty (2001), this circulation of the active biomass allows great expansion of the total amount of the biomass in the reactor and thus faster and more effective process. The biomass uses nutrients and oxygen to degrade organic matter (BOD) which serves as the electron donor. Oxygen is the most common electron acceptor which must be supplied to the process via aeration. Activated sludge process is an especially resourceful technique for remediating municipal wastewater which has typically steady flow characteristics and organic concentrations. The activated sludge is a complex matrix of myriad of different microorganisms which include characteristically bacteria and various different eukaryotes. Heterotrophic bacteria are typically the most dominant organisms and they are also the primary consumers of organic waste (Rittman & McCarty 2001).

The reliability and robustness of the activated sludge process relies on mixed microbial culture. Toxins, bacteriophages and other harmful factors may enter the process with wastewater and cause the death of certain microbial species in the sludge, but in the mixed

culture other microorganisms readily occupy the open ecological niche. Moreover, the number of different species allows the emergence of more specialised organisms which may utilise for growth only specific compounds that are not metabolisable by the majority of the community. (Rittman & McCarty 2001).

The activated sludge process is usually coupled with nitrification and denitrification processes. The nitrification process oxidises NH_4^+-N to nitrite NO_2^- and nitrate NO_3^-, whereas the denitrification is an anaerobic process in which nitrate and nitrite are reduced to N_2 gas. The nitrification is a mandatory process for some wastewaters because of the toxicity of ammonium to aquatic microorganisms and because of the high oxygen demand of ammonium. If the process includes denitrification, nitrification also serves as a required pre-treatment method to convert the nitrogen to utilisable form. Denitrification is used when the complete nitrogen removal is required, e.g. when discharging the effluent to waters that need protection against eutrophication or when the wastewater contains elevated NO_2^-/NO_3^- levels. (Rittman & McCarty 2001).

2.3 Microbial electrochemical systems

The first reported attempts to produce electricity with microbes were made as early as in 1910's by Potter (1911) and in early 1960's by Davis and Yarbrough (1962) who fed the microbes with hydrocarbons and glucose, even though the relation between living matter and electricity had been known for centuries (Galvani 1791). According to Du *et al*. (2007), the discovery that the current and power outputs of the MFCs were greatly enhanced with the addition of electron mediators re-evoked the interest in the technology in 1980's. In 1990's, microbes that can donate electrons directly to the anode without mediators were discovered by Kim et al. (1999) and that is considered as the real breakthrough of MFCs (Du et al. 2007).

Only the microbial electrochemical systems (MES) in which the produced current is used for electricity generation are called MFCs (Wang & Ren 2013). The current produced in alternative MESs can be used to power other interesting applications but addition of external electricity is required for the reactions to occur. Besides electricity production, MESs can be used to produce H_2 or other value-added compounds (Kuntke et al. 2014), remediate contaminants (Gregory & Lovley 2005), or the electrons can be used at the cathode to synthesise organic compounds or to drive desalination of water (Wang & Ren 2013). According to studies of Aelterman *et al*. (2006), current and voltage outputs can be enhanced by connecting several MFCs in series or in parallel with no obvious adverse effects on maximal power output.

There are three known ways of electron transport from the microbial cells to the anode electrode (Figure 2.1): direct electron transfer, mediated electron transfer, and transfer along nanowires. The mediators are compounds that transfer electrons from the organism

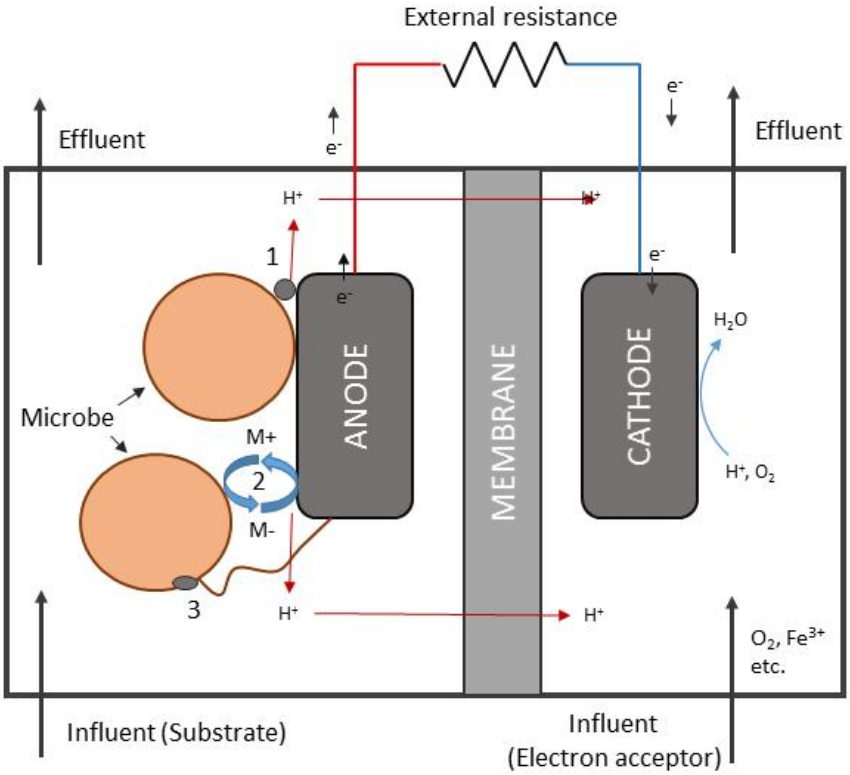

Figure 2.1 *Schematic picture of a MFC. Possible modes of electron transfer are (**1**) direct electron transfer via outer membrane cytochromes, (**2**) electron transfer through mediators, and (**3**) electron transfer via nanowires.*

to the electrode. The microorganism reduces mediators which are then oxidised in interaction with the electrode, leaving the electrons to the anode (Pant et al. 2010). The direct electron transfer is thought to function via terminal electron transport proteins found in the outer membranes of the microorganisms (Logan et al. 2006). The electrons are transferred from the substrate through the electron transfer chain in a series where the electron is always transferred from a protein in higher potential to a protein in lower potential, harvesting energy in every step. The anode electrode thus acts as the terminal electron acceptor instead of the terminal periplasmic protein, capturing the electrons and transferring them to the electric circuit (Lefebvre et al. 2011). Nanowires have been under rigorous study after their recent discovery by Reguera *et al*. (2005). Some bacteria, such as *Geobacter sulfurreducens*, are capable of forming highly conductive pili that the organism uses to transfer the electrons to the anode electrode (Malvankar & Lovley 2012). The nanowires are highly efficient and allow denser exoelectrogenic biofilm to the anode than direct electron transfer, because the organisms do not need to be as close to the electrode (Reguera et al. 2005).

2.3.1 Different MFC designs

A typical MFC consists of an anodic and the cathodic chamber which are separated by proton exchange membrane (Figure 2.1), but cathode may also be exposed directly to air to eliminate the need for a separate cathode chamber (Du et al. 2007). The reactors that include separate chambers for both the anode and the cathode are typically referred to as two-chamber MFCs, and the reactors from which the cathode chamber is eliminated may be referred to as one-chamber MFCs or air-cathode MFCs. The anode and cathode chambers can be constructed from various materials such as glass, polycarbonate and Plexiglas, whereas the electrodes themselves can be made from graphite, carbon-cloth, carbon paper, or platinum, among others (Du et al. 2007).

The membrane separating the chambers is used to allow the proton flow towards the cathode and to eliminate the diffusion of oxygen to the anode (Logan et al. 2006). According to Du *et al.* (2007), the proton exchange system affects the MFC's power output by affecting the internal resistance and concentration polarisation losses of the system, but a more recent report (Ge et al. 2014) claims that the membrane does not alter the power output significantly. Nafion (DuPont, USA) is popularly used because of high selectivity, but other alternatives exist such as other cation or anion exchange membranes, salt bridges, porcelain or the electrolyte itself (Du et al. 2007).

The usage of electrode materials, such as platinum and platinum black, improve the performance of the MFC as the activation polarisation losses are lower. Moreover, Pt has higher catalytic activity to oxygen than carbon-based materials. However other electrodes such as graphite, graphite felt and carbon-cloth are more affordable than expensive platinum. (Du et al. 2007).

According to Du *et al.* (2007), two-chamber MFCs are typically run in batch mode, even though continuous mode is also possible. They use chemically defined media and they are confined at the moment to laboratory scale only (Du et al. 2007). According to Du *et al.* (2007) the two-chambered MFCs are difficult to scale-up because of their complex design. Five typical two-chamber MFC designs are presented in Figure 2.2. Upflow MFCs (Figure 2.2d–e) were designed by Jang *et al.* (2004) for continuous operation: the anode was situated in the bottom of a cylindrical design through which the influent was circulated towards the cathode at the top of the cylinder. The effluent was recovered from the top of the cylinder. The anode and cathode chambers were partitioned with layers of glass wool and glass beads, respectively. (Jang et al. 2004). According to Du *et al.* (2007), the upflow MFCs are especially suitable for wastewater treatment because of the relative ease of their scale-up. As the reactors use fluid recirculation by energy-intensive pumping, the primary function of these reactors is the wastewater treatment and not electricity generation.

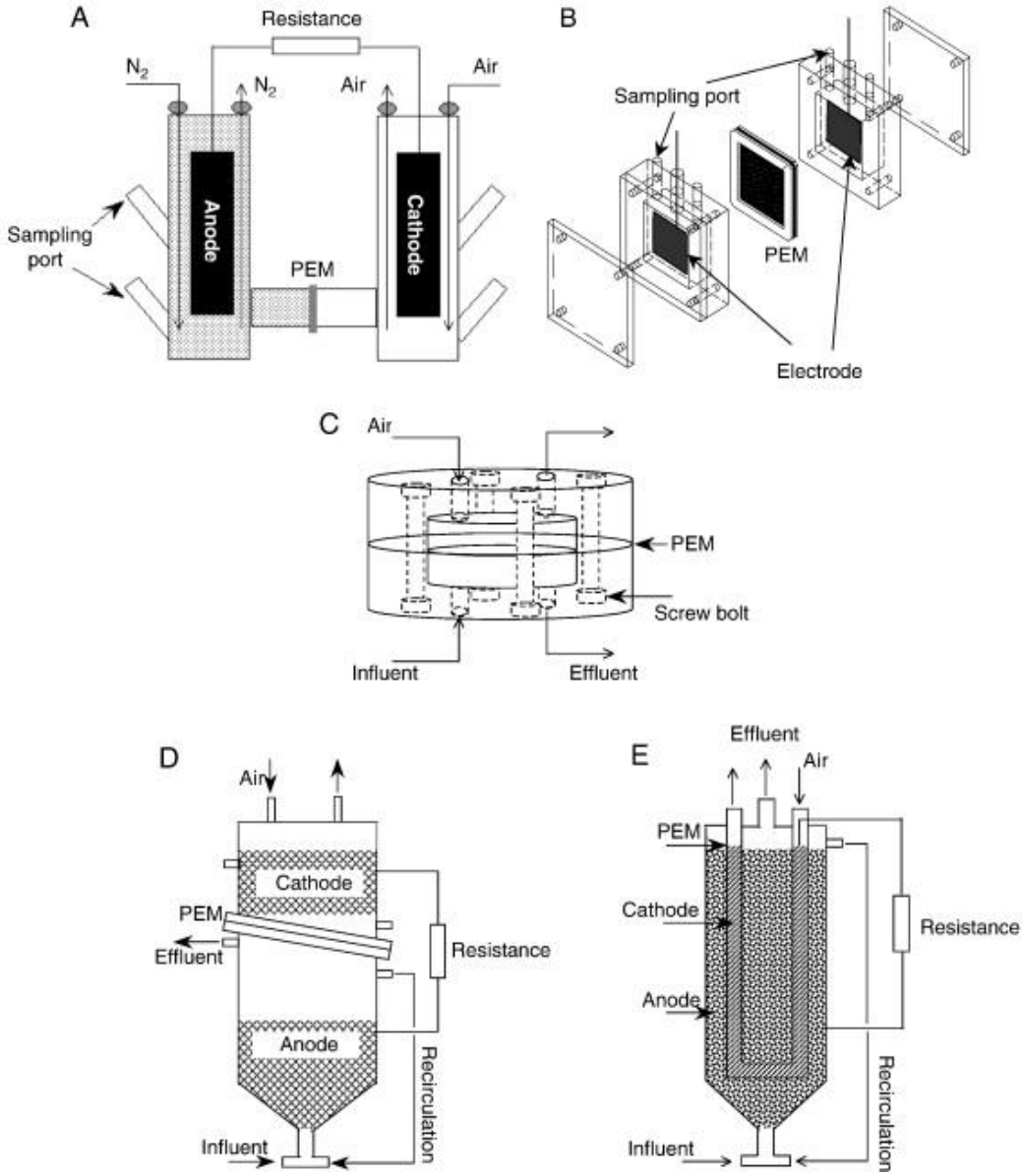

Figure 2.2 *Schematics of a two-compartment MFCs. (**a**) A typical cylindrical H-type MFC, (**b**) rectangular MFC, (**c**) miniature MFC of only 2 cm diameter, (**d**) upflow MFC, and (**e**) cylindrical MFC with U-shaped cathode compartment. Reprinted from Du et al. (2007), p. 469, Copyright (2007), with permission from Elsevier.*

The one-chamber MFCs are simpler to scale up than their two-chambered counterparts because of their simple design (Du et al. 2007). The cathode is adjoined to the anode chamber with a proton-permeable layer which allows the protons to pass on to the cathode (Logan et al. 2006). Tubular MFCs (Rabaey et al. 2005) contain the anode inside a tube-like design, whereas the cathode is situated on the outer case of the MFC. The cathode of the tubular MFC is dripped with the catholyte solution to keep the membrane from drying up (Rabaey et al. 2005).

2.3.2 Analysis of MFC performance

The analyses of the MFC performance are vital to understanding of the on-going processes in the MFCs. The most typical direct measurements from MFCs are those of cell voltage (*U*) and current (*I*) as they are easily obtained with data loggers. If the applied external resistance (R_{ext}) is known, power (*P*) can be derived from voltage data as follows.

$$P = \frac{U_{cell}^2}{R_{ext}} \quad (1)$$

However, because multiple different designs and applications of MFCs exist, the normalisation of the data eases comparisons between different designs. Typical normalisations include the power against anode area size (P_{An}, W/m^2) and power against the anode volume (P_v, W/m^3) which are commonly called the power density (Logan et al. 2006). Logan *et al*. (2006) thus insist that to be able to compare different MFCs, the relevant information such as the anode volume or area should always be reported to help the comparisons.

More recently Ge *et al*. (2014) have suggested the use of normalized energy recovery (NER) as the basis of power output reporting, especially for continuous systems. In NER, the power output of the MFC is compared to the treated amount of wastewater (Equation 2) or degraded COD (Equation 3) within a time window, whereas power densities are reported towards the reactor size. NER is reported as kilowatt-hours per cubic meter or as kilowatt-hours per kg COD. In the equations *t* stands for time, *V* the volume of wastewater treated during time *t*, *Q* the wastewater flowrate, and *ΔCOD* for the amount of COD degraded during time *t* in kg or kg/l. Although, the NER values have not been widely reported in recent articles.

$$NER = \frac{Pt}{V} = \frac{P}{Q} \quad (2)$$

$$NER = \frac{Pt}{\Delta COD(kg)} = \frac{P}{Q\ \Delta COD(kg/l)} \quad (3)$$

In addition to the whole cell voltages measured between the anode and the cathode, the electrode potentials can be measured. This requires a reference electrode that is positioned at the respective chamber. The electrode potentials are usually reported against normalised hydrogen electrode (NHE) which has a potential of 0 V. However, other reference electrodes are typically used for convenience such as Ag/AgCl (-0.197 V vs. NHE) and saturated calomel electrode (SCE, 0.241 mV vs. NHE). (Logan et al. 2006).

Cell electromotive force

The maximal theoretical cell voltage can be derived using Gibbs free energies of formation (ΔG_f^0) for oxidation-reduction reactions. The Gibbs free energy for a reaction in standard conditions is calculated as presented in Equation 4 (Thauer et al. 1977). The values of ΔG_f^0 are specific to each compound participating in the reaction.

$$\Delta G_f^0 = \Delta G_f^0(products) - \Delta G_f^0(reactants) \quad (4)$$

The overall electromotive force (emf), E_{emf}, of the cell is related to the Gibbs free energy as presented in Equation 5 (Logan et al. 2006). In the equation F represents the Faraday's constant ($9.64853 \cdot 10^4$ C/mol) and n the total amount of electrons produced per reaction.

$$E_{emf}^0 = -\frac{\Delta G_r^0}{nF} \quad (5)$$

The value of the Gibbs free energy in specific conditions (ΔG_r) can be calculated as presented in Equation 6

$$\Delta G_r = \Delta G_r^0 + RT\, ln(\Pi) \quad (6)$$

where R is the universal gas constant (8.31447 J mol^{-1} K^{-1}) and T the absolute temperature in Kelvins. Π is a unitless reaction quotient which can be calculated by dividing the activities of the products with those of the reactants. (Logan et al. 2006). As an example for the disproportionation of tetrathionate (Equation 16) the reaction quotient can be calculated per Equation 7.

$$\Pi = \frac{[S_4O_6^{2-}][H_2O]^6}{[S^0][SO_4^{2-}]^3[H^+]^{12}} \quad (7)$$

The direct equation for E_{emf} in specific conditions (Equation 8) can be derived by combining Equations 5 and 6 (Logan et al. 2006).

$$E_{emf} = E_{emf}^0 - \frac{RT}{nF}\, ln(\Pi) \quad (8)$$

According to Logan *et al.* (2006), Equation 8 is especially handy because it gives directly the maximal cell voltage when the reaction quotient is determined taking into account both the anodic and cathodic reactions. In addition, the feasibility of the reaction can be evaluated with Equation 8, E_{emf} being positive for an energetically favourable reaction. Using Equation 8 we can also determine theoretical anode and cathode potentials separately and combine them with Equation 9 where the negative sign results from oxidation reaction at the anode. The effects of different environmental conditions at anode and cathode potentials can be evaluated separately using Equations 8 and 9. This is helpful in

comparing e.g. how different cathodes or cathodic conditions, such as pH or substrate concentration, alter the whole cell voltage of the system.

$$E_{emf} = E_{cat} - E_{an} \quad (9)$$

Cell electromotive force does not take into account any internal losses which always reduce the maximal voltage in all electrochemical systems. Open circuit voltage (OCV) is the voltage that can be measured after a while from the cell when it has been detached to no current state (Logan et al. 2006). According to Logan *et al.* (2006), the OCV should theoretically approach cell's E_{emf}, but is usually somewhat lower due to various potential losses.

Coulombic efficiency

Coulombic efficiency is a term used to describe how large an amount (percentage) of the electrons available in the substrate are utilised to generate electricity. It is defined as the ratio of total Coulombs transferred from the substrate to the anode to maximum possible Coulombs from the utilised substrate. Coulombic efficiency is diminished when the microorganisms use alternate electron acceptors instead of the electrode. If the degradation reaction is known, the Coulombic efficiency can be calculated from the amount of generated electrical current and the amount of degraded substrate according to Equation 10. In Equation 10, ε_{Cb} is the Coulombic efficiency over time t, n_x the amount of substrate (moles) degraded in total, b_x the amount of electrons available from one mole of substrate, and F the Faraday constant. (Logan et al. 2006).

$$\epsilon_{Cb} = \frac{\int I\, dt}{n_x b_x F} \quad (10)$$

If wastewaters are used as the substrate, the Coulombic efficiency can be calculated against the amount of degraded COD as

$$\epsilon_{Cb} = \frac{M \int_0^t I\, dt}{F b V_{An} \Delta COD} \quad (11)$$

where M is the molecular mass of O_2 (32), b the amount of electrons exchanged per mole of oxygen (4), V_{An} the liquid volume of the anode and ΔCOD the change in COD over time t. (Logan et al. 2006).

Internal losses of the system

MFCs have always some internal losses which are typically classified to four groups according to their origin: ohmic losses, activation losses, mass transport losses, and metabolic losses (Logan et al. 2006). Interconnections, electrodes, membranes and low conductivity of used media cause ohmic losses which can be decreased by control of the connections and by the selection of the system components. Activation losses occur in

the molecular level, where redox reaction requires activation energy to happen, and they tend to increase at low currents. They can be reduced with large electrode surface area, improved electrode catalysis, high quality biofilm at the electrode, as well as increased operating temperature. Mass transport losses arise as the rate of mass transport limits current production. At high current densities the supply of reduced or oxidised metabolites to the electrode becomes the limiting factor. In poorly mixed systems the diffusional gradients run the show and thus the flux of the substrate to the biofilm plays a grand role. Mass transport losses can be seen as the rise of anode potential or the drop of cathode potential, depending on where the losses occur. Bacterial metabolic losses are caused by the innate need of the microorganisms to use the energy to run the cellular machinery and support life. (Logan et al. 2006).

The measured MFC voltage is typically linear compared to the system's current, which means that the total internal losses of the system can be derived from Equation 12, where R_{int} represents internal resistance that creates internal losses of the system (Logan et al. 2006). According to Cheng *et al*. (2006) the MFC systems provide maximal power output when their internal resistance is equal to applied external resistance.

$$E_{cell} = OCV - IR_{int} \tag{12}$$

The internal resistance of the system can be determined as the slope of the curve when the cell voltage is plotted against the current. Potentiostat can be used to control either the current or the cell voltage, allowing the examination of the system at that specific condition. In MFC studies, potentiostats are typically used in voltammetry tests in which the potential of the anode or the cathode electrode is altered at a certain scan rate. If the scan is going only in one direction, linear sweeping voltammetry (LSV) is in question, and if the scan continues to the reverse direction coming back to the starting potential, it is called cyclic voltammetry. Voltammetry is used to assess the electrochemical performance of microbial strains or cultures, delineating the standard redox potentials of redox active components and for piloting different cathode materials. (Logan et al. 2006).

Polarisation curves

Polarisation curves aid in the analysis and characterisation of fuel cells. According to Aelterman *et al*. (2008), periodical recording of the polarisation curves allows the monitoring of the development of the maximum current, the power generation, and the electrochemical properties of the anode. In the polarisation curve, the voltage is presented as a function of the current density and it can be generated for anode or cathode *an sich* or for the whole cell. Polarisation curves can be obtained with LSV, cyclic voltammetry or by altering the external resistance. Each curve has three distinct phases: (a) at zero current the voltage is at OCV, after which follows a steep decrease of voltage in the area where

activation losses dominate; (b) in the second phase voltage drops rather linearly with current, as ohmic losses dominate; and (c) quick voltage drop at high current as concentration losses are high. (Logan et al. 2006).

Power curve is obtained from the polarisation data when the power density is plotted against the current density. The curve begins from zero power, then peaks at maximum power point, after which power drops when the ohmic losses and electrode overpotentials increase to the point where no power is produced anymore.

2.3.3 MFC start-up strategies

The start-up of MFCs is a topic that has not yet been studied in great detail. However, two main strategies exist in MFC start-up: control with external resistance and control of anodic potential. Even though the comparisons of the MFC start-up between different poised anode potentials as well as different applied external resistances exist, only few studies have compared cross-wise between external resistances and controlled anode potentials. In a recent experiment (Kokko et al. 2015), MFCs started-up with high anode potential provided more current than those that were started-up with 100 Ω external resistance.

According to Lefebvre *et al*. (2011), the external resistance is a typical and perhaps the easiest way to influence the MFC operation. According to Aelterman *et al*. (2008), the applied external resistance can be chosen so that the MFC operates at desired anode potential and according to Lefebvre *et al*. (2011) live cell density at the anode of an MFC is inversely proportional to the applied external resistance. The external resistance may be adjusted by three distinct strategies: high resistance can be used to maximise the cell voltage, low resistance to maximise the current, or resistance may be matched to the internal resistance of the system to maximise the power output (Lefebvre et al. 2011). Lefebvre *et al*. (2011) found that applying low external resistance resulted in the highest power generation compared to the other two external resistance methods, most likely due to facilitated electron transfer which favoured the development of a bioelectrochemically active biofilm. Lower internal resistances were observed in the systems with low external resistance than in systems with varying or high external resistance. All the systems were able to produce the highest power when subjected to external resistance matching the internal resistance of the system. (Lefebvre et al. 2011).

As per Aelterman *et al.* (2008), the anode potential of an MFC affects the performance of the anode by determining how much energy is available for microbial growth. The energy gain of the microorganisms depends on the potential of the anode electrode compared to the standard biological reduction potential of the substrate. The greater the difference, the larger the amount of energy available to the organisms, thus increasing the amount of biomass in the system (Aelterman et al. 2008). The anode potential is thought to select for microorganisms respiratory proteins of which have more negative redox potentials

than the anode potential (Finkelstein et al. 2006; Wagner et al. 2010; Kokko et al. 2015). The organisms may not be able to donate electrons to the anode if the anode potential is allowed to decrease below that of the outer membrane protein or mediator responsible for the transaction (Wagner et al. 2010). Rabaey *et al*. (2004) have claimed that the transfer of electrons from the bacteria to the anode is the most critical step of the bioelectric process and that the bacteria which have electron chain components in the outer cell walls are more potentially well adapted to these systems. High anode potential is generally beneficial for the microbial culture because of higher amount of energy available for the organisms per molecule of substrate (Logan et al. 2006; Aelterman et al. 2008). Thus a positive anode potential is thought to increase the growth of microorganisms and result in quicker start-up of MFCs (Wagner et al. 2010). Yet to maximise the power output of a MFC for a certain current, the cathode potential of the cell should be as high and the anode potential as low as possible (Logan et al. 2006).

Contradictory studies can be found on the effects of anode potential on current generation and development of microbial community profile, as substrate, cell configuration as well as the inoculum may alter significantly the optimal anode potential of a MFC. As an example, Finkelstein *et al.* (2006) reported higher current output with more positive anode potential, whereas Torres *et al.* (2009) obtained the opposite results. Yet according to Wagner *et al*. (2010) and Kokko *et al.* (2015), more often than not applying positive anode potential has led to shorter start-up times, improved bacterial growth as well as more diverse exoelectrogenic bacterial community. Wagner *et al*. (2010) state that there is no accepted method to define the optimal anode potential for a MFC. The main paradigm of anode potential control is that setting a poised high anode potential creates quite different environment compared to those that naturally develop in MFCs. As the biofilm develops at the anode, the potential of a non-controlled anode becomes increasingly negative over time before reaching a plateau, value of which depend on the used substrate, cathode, current density as well as external resistance. (Wagner et al. 2010).

Zhu *et al*. (2014) claim that when using *Geobacter sulfurreducens* as inoculum, the maximum current production escalated with raising poised anode potential until 0.21 V (vs. NHE), after which no improvement was observed. Furthermore, they (Zhu et al. 2014) claim, that set anode potentials do not change the microbial community, contradicting the results of Commault *et al*. (2013) and Torres *et al*. (2009) that anode potential can be used to select between *Geobacter* strains.

2.3.4 Common wastewater components as MFC substrates

According to Logan *et al.* (2006) it is important to be able to evaluate the MFC performance in terms used in wastewater treatment, as MFCs have been proposed as a wastewater treatment method. According to them, at least the overall performance should be evaluated in terms of BOD, COD, or total organic carbon removal. Coulombic and energy calculations can also be performed with regard to COD removal which is a typical

measure in wastewater treatment. (Logan et al. 2006). Even though MFCs are considered a promising option for wastewater treatment because of their ability to function in relatively low strength waters (Logan et al. 2006), and to degrade highly complex pollutants (ElMekawy et al. 2016), pure substrates have been found to generate increased power and energy recovery compared to complex substrates found usually in wastewaters (Ge et al. 2014).

Various types of wastewaters that contain organic compounds have been used as MFC substrates, including domestic and industrial wastewaters. Domestic wastewater has been treated in MFCs with 83% COD removal and 18% Coulombic efficiency (Cusick et al. 2010). Brewery wastewater has been used in multitude of MFC studies because of its low strength and recalcitrance (Feng et al. 2008), but was found, among bakery wastewater, to generate less current (10 mA/m^2) than forest (125 mA/m^2) and dairy (25 mA/m^2) industry wastewaters (Velasquez-Orta et al. 2011). Zhuang *et al*. (2012) were able to obtain 176 W/m^2 power density from a tubular air-cathode MFC in conjunction to 84% COD and 91% NH_4^+-N removal from real swine wastewater.

Acetate has been one of the most used substrates for electricity generation in MFCs, but it is a very simple substrate which is used much more easily than most wastewaters (Pant et al. 2010). According to Pant *et al*. (2010) it is used commonly to benchmark new MFC technologies, designs and components. Chae *et al*. (2009) found that acetate was better substrate in terms of Coulombic efficiency as well as power output compared to butyrate, propionate and glucose. Acetate has also been used to amend wastewater in the start-up phase to enhance the electricity generation (Min & Angelidaki 2008).

Glucose is a fermentable hexose sugar utilised by most forms of life which can be found in e.g. starch processing and forest industry wastewaters. Chae *et al*. (2009) found that glucose provided the lowest Coulombic efficiency among the tested compounds because of enrichment of competing non-electrogenic bacteria. The growth of the competing bacteria allowed, however, later-on wider range of substrates than the other tested compounds as a result of a more complex biofilm (Chae et al. 2009). According to Pant *et al*. (2010), as glucose is a fermentable substrate it is metabolised also through routes that are not electrogenic such as fermentation and methanogenesis.

Xylose is a pentose sugar that is the main component of hemicellulose xylan, one of the building blocks of biomass. It is rich in forest industry wastewaters (Pokhrel & Viraraghavan 2004) and woody biomass hydrolysates (Vilela et al. 2015), but its industrial usage has been hindered as it is not fermentable to alcohol by *Saccharomyces cerevisiae* without a significant degree of genetic engineering (Vilela et al. 2015). Catal *et al*. (2008) suggested lignocellulosic-biomass derived monosaccharides as potential MFC substrates after testing electricity production from 12 different monosaccharides. They found out that xylose produced the highest power density (2330 mW/m^2) of the tested pentoses, which exceeded also that of glucose (2160 mW/m^2), the best-performing hexose (Catal

et al. 2008). Among the compounds they tested, only glucuronic acid produced higher power density (2770 mW/m^2) than xylose. In more recent studies using xylose as the substrate, Li and co-workers (2014) were able to obtain 2625 mW/m^3 power density with *Ochrobactrum* sp. 575 and Mäkinen and co-workers (2013) observed 590 mW/m^2 power density with a compost enrichment culture.

To the author's knowledge, only sulphur compounds have been used as inorganic substrates in MFCs. Reduced inorganic sulphur compounds (RISCs) can be found from process waters of sulphide mineral processing facilities (Liljeqvist et al. 2011). RISCs are biologically degraded in the environment producing acidity, which may enhance dissolution of metals if let to happen uncontrollably, and thus the RISC containing wastewaters must be treated before their run-off to the nature (Sulonen et al. 2016). Rabaey *et al*. (2006) used MFCs to oxidate sulphide to elemental sulphur, producing 47 W/m^3 power output, and tetrathionate has been degraded in MFCs producing 26 mW/m^2 (Sulonen et al. 2015) and 2.5 mW/m^2 (Ni et al. 2016) power densities. Furthermore, Zhang *et al*. (2014) have used *Desulfuromonas* strain TZ1 to oxidate sulphur to sulphate and generate current in MFCs.

2.4 Harnessing extremophilic life to power MFCs

Life in extreme physical and geochemical conditions, such as high and low temperatures, high metal concentrations or extreme nutritional limitations, which are hostile to most forms of life is considered extremophilic (Dopson et al. 2016). Taxonomically, extreme environments are defined as environments with low species diversity and lack of whole taxonomic groups (Bott & Brock 1969). Such environments are found in nature e.g. from hot springs and geysers, deep sea bottoms and hydrothermal vents, salt flats and lakes, evaporates, deserts, the arctic, and the atmosphere (Rothschild & Mancinelli 2001). They can be as well originating from anthropogenic action such as acidic mine waters with extreme metal concentrations or nuclear sites with high radioactivity (Dopson et al. 2016).

Extremophilic microorganisms have been in the interest of researchers in the recent years because of their huge biotechnological potential arising from highly specialised biochemical pathways and enzymes (Morozkina et al. 2010). Extremophilic microorganisms have been used in various applications in biotechnology, medicine and industry. According to Morozkina *et al*. (2010) the applications range from resistance increase in agriculture and bioremediation of soils to production of bioethanol and biopolymers, and to development of biosensors and extremoenzymes (Morozkina et al. 2010).

2.4.1 Extremophilic microbial fuel cells

Extremophilic microorganisms have been used to power up MFCs because of their adaptation to operation conditions and substrates that are hostile to most forms of life. Extreme

environments used in MFCs include high acidity and alkalinity, high and low temperature, and high salinity (Dopson et al. 2016). The organisms living in these conditions are called acidophiles, alkaliphiles, thermophiles, psycrophiles, and halophiles, respectively.

According to Dopson *et al*. (2016) organisms are acidophiles if they have growth optimum at pH lower than 5. Several acidophiles are capable of ferrous iron, sulphur, RISC and organic carbon oxidation to support growth. As they typically catalyse metal sulphide dissolution, they often exhibit high tolerance to very high metal concentrations. Only few studies, in which the performances of MESs in extreme conditions are compared systematically, have been conducted. The comparison is also challenging as the different MES architectures and choices of electrolytes impact the observed performance. The pH of the anodic environment alters directly the attainable cell voltage, and ascending cell voltage can be observed in elevating pH. Generally, acidophiles show efficient bioelectrochemical performance with high oxidation rates of the substrates to produce current. (Dopson et al. 2016). According to Borole *et al*. (2008), one of the advantages of acidophilic MFCs is that the high proton concentration at anode leads to increased proton gradient towards the cathode, increasing the availability of protons at the cathode and thus enhancing cathodic reactions. They do however claim that this is true only to systems with high power densities. As per Dopson *et al*. (2016), the change in cell pH can have a notable effect on MFC performance, e.g. as much as 0.6 V when oxidising acetate in environments with pH 2 and 10.

Acidophilic MFCs have utilised various different electron donors and inocula. According to a recent review on the subject by Dopson *et al*. (2016), typical pure culture microorganisms have been *Acidithiobacillus ferrooxidans*, *Acidiphilium* spp., and *Acidiphilium cryptum*, whereas mixed cultures have been obtained from anaerobic sludge, sediments and different kinds of wastewaters. The utilised electron donors have included organic carbon compounds, such as acetate and glucose, as well as wastewaters and tetrathionate. Utilised pH values have ranged 2.0–4.7. (Dopson et al. 2016). Moderately acidic environments (pH 4–5.5) have been used to treat organic wastewaters with inocula from neutral environments (Kim et al. 2014). Sulfate have been removed from acidic wastewaters with a MES that used organic carbon-fed microbes to reduce sulphate to sulphide which was electrochemically oxidized to elemental sulphur on the anode surface (Liang et al. 2013; Zheng et al. 2014). A MFC using tetrathionate in pH 2.0 was used to treat simulated mining wastewater, utilizing *A. ferrooxidans* and *Ferroplasma acidiphilum* co-culture as effective organisms (Sulonen et al. 2015). Another approach was taken by ter Heijne *et al*. (2007) who used *A. ferrooxidans* to regenerate ferric iron at the cathode and observed 38% increase in power output, most likely due to elevated Fe^{3+} concentration.

Even though alkaline environments provide more energetically favourable processes than acidic media, acidifying reaction at the anode chamber means that the anode is often working in an almost neutral environment, even though the influent is alkaline (Dopson

et al. 2016). According to Dopson *et al*. (2016) the typical inocula in alkaline MFCs include *Bacillus* spp., *Bacillus pseudofirmus*, *Pseudomonas alcaliphila*, and *Corynebacterium* sp., as well as mixed cultures from composts, wastewaters, and different types of sludges. The most commonly utilised electron donor has been acetate, but also other organic substrates, synthetic wastes and urine have been used (Dopson et al. 2016). Alkaliphilic MESs have been used for ammonium recovery and hydrogen production by Kuntke and co-workers (2014). Typical pH range in alkaliphilic MFCs has been 9.0–11.0 (Dopson et al. 2016).

The temperature has a great impact on the MFC performance as it impacts the microorganisms, the electrochemical reactions, as well as the Gibbs free energy change in the reactions. All the microorganisms have an optimum temperature of growth in which they perform in the most effective way. Generally, chemical reactions as well as the enzyme activity is enhanced in higher temperatures (Morozkina et al. 2010). Both high and low temperature MFCs have been developed, because for example low temperature MFCs allow direct use of low-temperature wastewaters as the substrate and high temperatures offer lowered risk of contamination, higher substrate solubility and high rates of mass transfer (Dopson et al. 2016) as well as faster degradation processes (Carver et al. 2011). According to Dopson *et al*. (2016), lowering the operating temperature of the MFC has many downsides, as it reduces the power and current (Larrosa-Guerrero et al. 2010), start-up time (Cheng et al. 2011), as well as substrate oxidation (Larrosa-Guerrero et al. 2010). The advantage of running the MFC in the low temperature is higher Coulombic efficiency (Michie et al. 2011; Dopson et al. 2016), whereas the downside of thermophilic MFCs is the increased evaporation. The temperatures in thermophilic MFCs have ranged 50–60 °C and in psychrophilic MFCs 4–15 °C (Dopson et al. 2016). Acetate has been the most common substrate choice in thermophilic systems (Wrighton et al. 2011; Fu et al. 2013; Parameswaran et al. 2013) but more variation is found in psychrophilic systems where acetate (Xu et al. 2014), wastewater (Larrosa-Guerrero et al. 2010; Michie et al. 2011), and organics (Holmes et al. 2004; Catal et al. 2011) have been commonly used. Pure culture inocula such as *Thermincola ferriacetica* (Parameswaran et al. 2013), *Thermincola potens* (Wrighton et al. 2011) and *Calditerrivibrio nitroreducens* (Fu et al. 2013), as well as anaerobic digester cultures (Ha et al. 2012; Fu et al. 2013) have been used in thermophilic MFCs, whereas psychrophilic MFCs have been inoculated with sediment (Holmes et al. 2004; Larrosa-Guerrero et al. 2010), wastewater (Catal et al. 2011), and sludge cultures (Michie et al. 2011; Xu et al. 2014).

Highly saline environments have generally a positive effect to the MFCs, as the high salinity decreases the internal resistance of the systems by increasing solution conductivity. Furthermore, the proton diffusion resistance is decreased if salinity is provided with buffering compounds. However, addition of salts to the medium is unsustainable and costly but practical applications could be developed to environments with naturally high salt concentrations. (Dopson et al. 2016). Halophilic MFCs have been studied in NaCl

concentrations ranging 18–158 g/l, mostly with acetate as the substrate (Liu et al. 2008; Miller & Oremland 2008; Abrevaya et al. 2011; Miceli et al. 2012; Dopson et al. 2016). The inocula have been various as different wastewater cultures (Liu et al. 2008; Lefebvre et al. 2012), environmental samples from saline environments (Miller & Oremland 2008; Miceli et al. 2012), as well as pure cultures of *Geoalkalibacter subterraneus* (Carmona-Martinez et al. 2013) and *Haloferax volcanii* (Abrevaya et al. 2011) have been used.

According to Dopson *et al.* (2016) the research regarding extremophilic MESs has just begun and the research of different combinations of inocula and substrates will reveal new possibilities for applications. They claim that the key research need is in the discovery of extremophilic electricigens that can treat wastewaters. One of the studies in which new electricigens were searched from highly acidic mining process water found that *A. ferrooxidans* and *F. acidiphilum* were able to degrade tetrathionate as a co-culture in MFCs and produce 13.9 mW/m^2 power density (Sulonen et al. 2015). These two extreme acidophiles are presented in more detail in the following sections.

2.4.2 *Acidithiobacillus ferrooxidans*

A. ferrooxidans is a well-studied extremophilic bacterium first characterised by Temple and Colmer (1951) as *Thiobacillus ferrooxidans*. Kelly and Wood (2000) reclassified the organism to the genus *Acidithiobacillus* as they separated the notably diverse genus *Thiobacillus* to three different genera (*Acidithiobacillus*, *Halothiobacillus* and *Thermithiobacillus*). *A. ferrooxidans* is an obligate acidophile, facultative aerobe (Pronk et al. 1992), as well as an obligate chemolithoautotroph. It is a Gram-negative γ-proteobacterium, with rod-like shape, the optimal temperature of growth of 30–35 °C (limit 10–37 °C), and optimal pH of 2.5 (limit 1.3–4.5). The type strain of the species is ATC 23270, but genetic variation inside the species is large. (Kelly & Wood 2000).

By definition *A. ferrooxidans* oxidises reduced sulphur compounds (RISCs) and ferrous iron for autotrophic growth (Kelly & Wood 2000; Méndez-García et al. 2015). Other known utilised electron donors include molecular hydrogen (Drobner et al. 1990; Ohmura et al. 2002) and formic acid (Pronk et al. 1991). The organism can utilise oxygen, ferric iron, as well as elemental sulphur as the terminal electron acceptor (Ohmura et al. 2002). It fixes carbon via Calvin cycle (Unz & Lundgren 1961) as well as elemental nitrogen (N_2) which makes it a primary producer in acidic environments (Valdés et al. 2008; Méndez-García et al. 2015). Yet according to Méndez-García *et al.* (2015), nitrogen is fixed with mediation of the Mo-Fe nitrogenase enzyme complex, activity of which is sensitive to the presence of oxygen, which thus has to occur in anoxic conditions. *A. ferrooxidans* is an important iron reducer and oxidiser in acidic habitats. It possesses a super-complex which is capable of iron oxidation and oxygen reduction (Castelle et al. 2008; Méndez-García et al. 2015). According to Méndez-García *et al.* (2015), it is likely that in anaerobic conditions the organism uses iron as terminal electron acceptor, coupling its reduction to sulphur and hydrogen oxidation, a view supported by experiments of Pronk *et al.* (1992).

A. ferrooxidans is a key organism in various bioleaching applications because of its rather unique feature of being capable to harness energy by oxidising ferrous iron to ferric form in the acidic environments (Valdés et al. 2008). It has been widely used industrially to solubilise metal sulphides found in ores (Beard et al. 2011). Therefore, it has been widely studied not only for industrial biomining applications but also for microbial electrochemical systems. Its ferrous iron oxidation pathway has been studied in detail which makes it a promising microbe for utilisation in the MESs. The organism can be used to regenerate ferrous iron to ferric iron in the cathode compartment of a microbial fuel cell. According to Valdés *et al.* (2008), *A. ferrooxidans* creates a reverse electron flow from Fe^{2+} to NADH exploiting the low extracellular pH. In aerobic environment, it couples Fe^{2+} oxidation to O_2 reduction but uses Fe^{3+} as the energy pool in anoxic conditions to oxidise various substances such as S^0, NAD^+ and H_2, to gain energy (Valdés et al. 2008). According to Méndez-García *et al.* (2015), *A. ferrooxidans* uses electron transport chain to transfer electrons from reduced species to most likely outer membrane cytochrome *c* to generate anabolic energy.

In contrast to a well characterised iron-oxidising system, there are several pathways that are proposed for reduced sulphur compound oxidation in *A. ferrooxidans* (Valdés et al. 2008; Quatrini et al. 2009; Kucera et al. 2016). RISC oxidation provides the organism a process that is energetically more favourable than that of reducing iron but requires special enzymatic pathways (Méndez-García et al. 2015). The sulphur oxidising system of *A. ferrooxidans* is scientifically interesting as the organism lacks the Sox system found in many other extreme acidophiles, including other *Acidithiobacilli*, which can oxidise RISCs directly to sulphate without sulphite as an intermediate (Méndez-García et al. 2015). Early studies of Eccleston and Kelly (1978) suggested that the organism oxidises tetrathionate ($S_4O_6^{2-}$) to sulphate (SO_4^{2-}) firstly through thiosulphate ($S_2O_3^-$) and then via sulphite (SO_3^-) and elementary sulphur. More recently it has been shown that *A. ferrooxidans* possesses tetrathionate hydrolase (TetH), a key enzyme in tetrathionate degradation, which hydrolyses tetrathionate to produce thiosulphate which thiosulphate quinone oxidoreductase (DoxDA) then oxidises further (Quatrini et al. 2009; Kanao et al. 2007). According to Kanao *et al.* (2007), all the reactions of RISCs may not need enzymatic catalysation as they, especially polythionates, are chemically very reactive. Therefore, the precise mechanism of the degradation still remains unclear. Thiosulphate is known to be unstable in acidic conditions and decomposes eagerly to sulphite and elementary sulphur. In addition, sulphite reacts with ease with protons of acidic solutions to produce sulphur dioxide and water (ILO 2009).

According to the bioinformatics studies (metabolic reconstruction accompanied with transcript profiling) conducted by Quatrini *et al.* (2009), the aerobic sulphur oxidation system uses a second bc_1 complex that is able to transfer electrons directly from sulphur to oxygen. The authors claimed that the bc_1 complex receives electrons from the quinol pool and releases them to the membrane bound enzyme cytochrome c_4 CycA2 or Hip, a

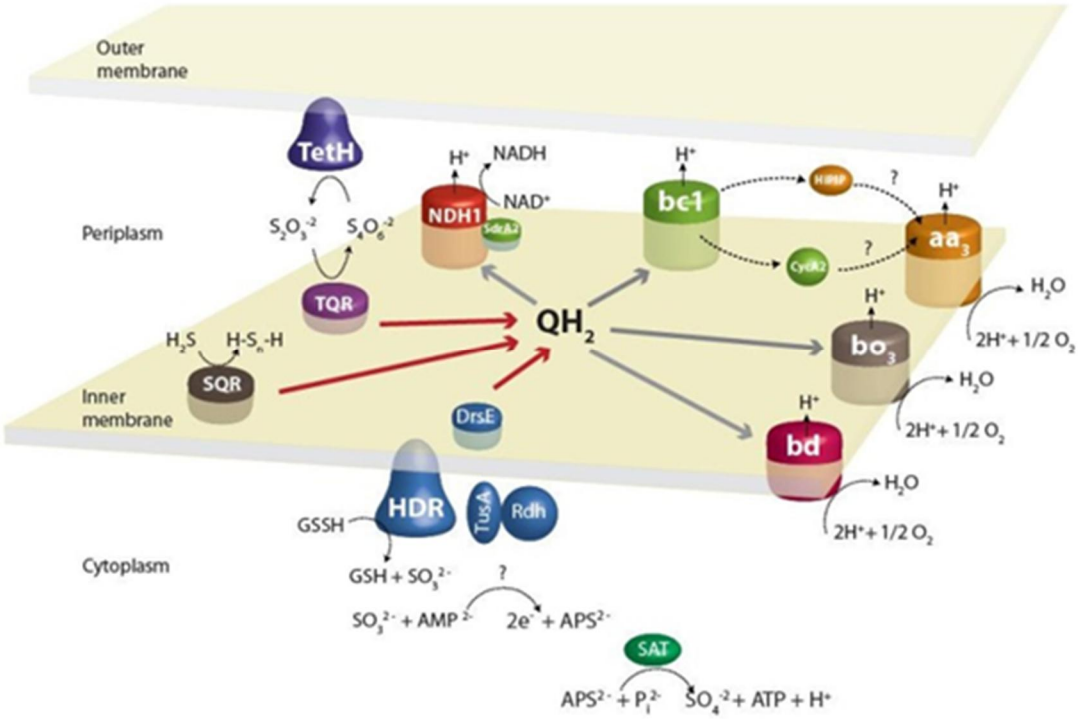

Figure 2.3 *Model of sulfur oxidation in* A. ferrooxidans *ATCC 23270. QH_2, quinol pool;HDR, heterosulphide reductase; TetH, tetrathionate hydrolase; TQR, thiosulphate quinone reductase; SQR, sulphate quinone reductase; SAT, ATP sulphurylase. Reprinted under CC BY 2.0 from Quatrini* et al. *(2009).*

high potential iron-sulphur protein. The involvement of this mechanism is consistent with models of aerobic and anaerobic oxidation of sulphur and formate studied by Pronk and co-workers (1991) and earlier branched electron flow models (Brasseur et al. 2004; Bruscella et al. 2007; Quatrini et al. 2006) of S^0 oxidation presented in Figure 2.3 (Quatrini et al. 2009). Additionally, backing up the model, sulphur oxidation has been suggested to take place in the cytoplasmic space (Silver & Lundgren 1968) and has been shown to be inhibited by respiratory chain inhibitor HQNO (Corbett & Ingledew 1987).

The capability of *A. ferrooxidans* to anaerobic growth was verified with cultures grown in elemental sulphur and ferric iron (Pronk et al. 1992). In their study, Pronk *et al.* (1992) found that the organism was able to couple ferric iron reduction to the oxidation of elemental sulphur to support growth but the absence of either of these substrates halted it. They observed also that the concentrations of the reactants altered as per Equation 13 which was suggested as the metabolic total reaction of anaerobic sulphur oxidation.

$$S^0 + 6\,Fe^{3+} + 4\,H_2O \rightarrow H_2SO_4 + 6\,Fe^{2+} + 6\,H^+ \qquad (13)$$

Even though the anaerobic growth was possible, the achieved yields were low as the concentration of ferric iron limited growth in batch cultures. The experiment emphasizes the assumptions (Pronk et al. 1991; Quatrini et al. 2009) that the ferric iron reducing enzyme system receives electrons from the ferrous iron oxidoreductase and that the produced

electrons are used through a common quinol pool. The organism is not only able to consume elemental sulphur but is also capable of producing elemental sulphur in oxygen deprived conditions (Beard et al. 2011). Further according to Beard *et al*. (2011), TetH is assumed to be secreted to the extracellular matrix and it is also functional in anoxic conditions where it can catalyse the hydrolysis of tetrathionate to disulphane monosulphonic acid (DSMSA; $HS_2SO_3^-$) and sulphate. DSMSA reacts with other DSMSA molecules to produce polythionates and water in aerobic conditions or polythionates and hydrogen sulphide in anoxic conditions.

2.4.3 *Ferroplasma acidiphilum*

Ferroplasma acidiphilum is a relatively recently discovered (Golyshina et al. 2000) hyperacidophilic archaeon found in acid mine drainage environments. It belongs to the order of *Thermopalsmatales* which comprises of organisms that lack cell wall, have a single membrane bounding cytoplasm, and exhibit pleomorphic shapes (Méndez-García et al. 2015). *F. acidiphilum* is a chemolithotrophic or mixotrophic (Bonnefoy & Holmes 2012) and strictly aerobic organism (Golyshina et al. 2000). It has an optimal pH for growth in 1.7 and it can grow on pH range 1.3–2.2. Growth can be observed in temperature range of 15–45 °C with temperature optimum at 35 °C. (Golyshina et al. 2000).

F. acidiphilum's proteins contain unusual amounts of iron which has been suggested to aid in protein stabilisation in low intracellular pH (5.5) but which also subjects the organism to oxidative damage (Ferrer et al. 2007; Bonnefoy & Holmes 2012). According to Golyshina *et al.* (2000) and Dopson *et al.* (2005), *Ferroplasma spp.* are iron oxidisers capable of chemo-organotrophic growth on yeast extract. It has also been suggested that *Ferroplasmas* may prefer heterotrophic lifestyle (Tyson et al. 2004). According to Tyson *et al.* (2004), the organism does not fix nitrogen but it is assumed to obtain nitrogen fixed by other microorganisms with ammonia and amino acid uptake. No elemental or reduced sulphur compound oxidation has been observed with *F. acidiphilum* (Golyshina et al. 2000), unlike with the related order *Sulfolobales*.

With exception to *Acidithiobacilli*, only limited knowledge is available on the metabolic processes of iron oxidation in acid mine drainage habitats. According to a recent study (Castelle et al. 2015), the iron oxidation pathway in *F. acidiphilum* is distinct from other iron oxidisers found in the same environments and may constitute of two complexes (Figure 2.4). The first complex is a forward electron flow complex (so-called downhill pathway) which is able to oxidise ferrous iron by reducing molecular oxygen and generating ATP in the process with sulfocyanin as the primary electron acceptor. Similar model is also presented for related archaeon *Ferroplasma acidarmanus* (Dopson et al. 2005). The second complex is a reverse electron flow complex which is a putative homologue of a Rieske/cytochrome *b*-like complex and is proposed to enable the regeneration of reducing power as NADH or NADPH. Castelle *et al.* proposed that the uphill pathway is possible if it is chemiosmotically coupled to the downhill pathway. (Castelle et al. 2015).

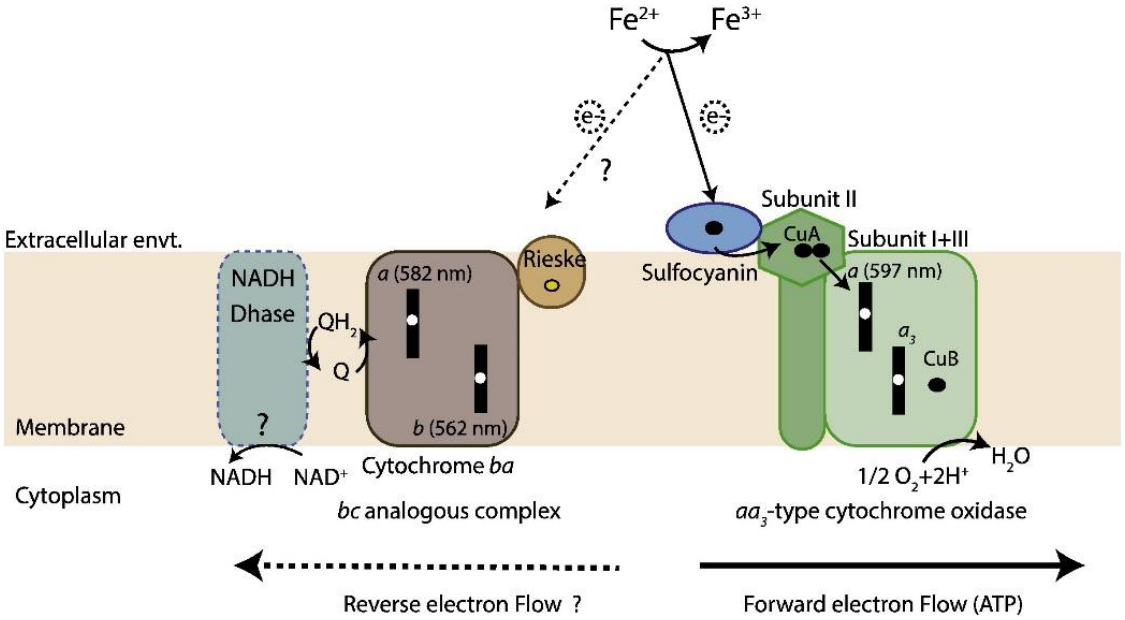

Figure 2.4 *Tentative model of ferrous iron oxidation pathway in* F. acidiphilum. *The forward elecron flow (downhill pathway) encompasses sulfocyanin and* aa_3*-type heme-copper oxidase, while the reverse electron flow (uphill pathway) may comprise a complex homologous to a Rieske/cytochrome* b*-type complex and a putative NADH dehydrogenase. Q, quinone;* QH_2*, quinol; env., enrivonment. Reprinted from Castelle* et al. *(2015), p. 727, Copyright (2015), with permission from Elsevier.*

3. MATERIALS AND METHODS

This study consists of three distinct experiments in which the suitability of MFCs to treat simulated wastewaters was assessed. Two experiments aimed to degradation of reduced inorganic sulphur compounds from synthetic mining wastewater with pure cultures of *A. ferrooxidans* and *F. acidiphilum* and one to lower chemical oxygen demand of synthetic forest industry wastewater. The suitability of *A. ferrooxidans* and *F. acidiphilum* to degrade tetrathionate was first tested with aerobic batch bottle experiments and after that in anaerobic two-chamber fed-batch MFCs. In the last experiment a microbial consortium was used to degrade organic material in synthetic wastewater in flow-through open-air cathode MFCs.

3.1 Tetrathionate degradation in aerobic batch cultures

Aerobic tetrathionate degradation by the pure microbial cultures of *A. ferrooxidans* and *F. acidiphilum* was assessed with batch bottle experiments. The growth medium contained 10% (v/v) mineral salts medium (MSM) and 1% (v/v) trace element solution (TES). Potassium tetrathionate ($K_2S_4O_6$) was added as the substrate to final concentration of 2.5 g/l using a sterile-filtered tetrathionate stock solution containing 50 g/l $K_2S_4O_6$ and having pH of 2.0. MSM was sterilised in autoclave (121 °C, 20 min) and TES by sterile-filtering. The compositions of the solutions were as presented in Table 3.1. The pH of all the solutions was set to 2.0 with sulphuric acid. The cultures that contained solely *F. acidiphilum* were also supplemented with 0.225 ml sterilised yeast extract (10% w/v) because the organism as a heterotroph cannot survive without an organic carbon source. Yeast extract provides thus the organism a source of carbon it can use for growth. The effective volume of the cultivations was 150 ml and they were carried out in 250 ml Erlenmeyer flasks. The caps of the flasks were loosely tightened to ensure the availability of oxygen. The batch bottles were cultivated in ambient temperature (20±2 °C) under 150 rpm agitation.

The batch bottles were sampled 2–3 times a week to gather data from the substrate usage ($S_4O_6^{2-}$), the end metabolites (SO_4^{2-}), pH and the biomass concentration. New batches were started from the old bottles when the $S_4O_6^{2-}$ concentration fell below 0.5 g/l to prevent possible lysis of the cells in the absence of the substrate in the applied extreme conditions where cells require energy to maintain favourable intracellular pH. All the cultivations were renewed at the same time when the first culture had reached the aforementioned substrate concentration.

Sample of 5.5 ml was taken under laminar flow from each cultivation bottle. Part of the sample (4 ml) was immediately used to measure optical density. The rest (1.5 ml) of the

Table 3.1 *The compositions of the solutions used in media preparation.*

Trace element solution (TES)	c (mg/l)	Mineral salts medium (MSM)	c (g/l)
$FeCl_3 \cdot 6\ H_20$	11	$(NH_4)_2SO_4$	3
$CuSO_4 \cdot 5\ H_20$	0.5	KCl	0.1
H_3BO_3	2	K_2HPO_4	0.5
$MnSO_4 \cdot H_20$	2	$MgSO_4 \cdot 7\ H_20$	0.5
$Na_2MoO_4 \cdot 2\ H_20$	0.8	$Ca(NO_3)_2 \cdot 4\ H_20$	0.013
$CoCl_2 \cdot 6\ H_20$	0.6		
$ZnSO_4 \cdot 7\ H_20$	0.9	Tetrathionate stock	c (g/l)
Na_2SeO_4	0.1	$K_2S_4O_6$	50

sample was first centrifuged with Eppendorf Centrifuge 5417R (Eppendorf, Germany) with 7500 rpm in ambient temperature (20±2 °C) for 10 minutes to separate the cell material from supernatant. The supernatant was filtered with Chromafil Xtra PET-20/25 0.2 µm sterile filter (Macherey-Nagel, Germany) to dispose of any residual cell material and stored in -20 °C for the analyses.

The cultivations were enriched by inoculating new batch bottles from the previous batch rounds. The first bottles were inoculated from the cultures that were grown in DMSZ media described later in Section 3.4. Two parallel cultivations of solely *A. ferrooxidans* and *F. acidiphilum*, two binary cultivations containing both organisms, and an abiotic control were used in six enrichment rounds in total.

Two separated cultures, denoted A and B, were used to inoculate the first aerobic enrichments. In the first round, the cultures were pure cultures of *A. ferrooxidans* A and B, and *F. acidiphilum* A and B. Used binary cultures, containing both organisms, were denoted as 'Binary culture A' and 'Binary culture B', containing *A. ferrooxidans* A and *F. acidiphilum* A, and *A. ferrooxidans* B and *F. acidiphilum* B, respectively. After the first enrichment round the next bottles were inoculated from the corresponding bottle in the previous round. However, in the third batch round, the Binary culture B and *A. ferrooxidans* B cultures were started from the first round bottles because of insufficient growth during the second enrichment. The same cultures were inoculated from the second round bottles for the fourth round, as the culture revived during third enrichment and gained almost the same phase with other batches.

In the last (sixth) enrichment round 10% (v/v) of phosphate buffer (1.0 M) was added to the medium to reduce the effect of proton formation on medium pH. The buffer stock was prepared from H_3PO_4 and KH_2PO_4 with 0.466:0.534 concentration ratio to produce a buffer with a pH of 2.11. The phosphate buffer was used because of well matching pK_a value (2.15) to the medium pH which should allow optimal buffering at low pH values observed in the experiments.

3.2 Tetrathionate degradation in microbial fuel cells

The capability of the pure cultures of *A. ferrooxidans* and *F. acidiphilum* to degrade tetrathionate and produce current in MFCs was studied using two parallel cultivations of pure *A. ferrooxidans* cultures as well as two binary cultivations containing both *A. ferrooxidans* and *F. acidiphilum*. In addition, an abiotic (inoculum replaced with sterile MQ water) and biotic (inoculated with a mixed culture known to produce electricity from tetrathionate) control MFCs were used. No MFCs with solely *F. acidiphilum* were started as the results from aerobic batch bottle experiments showed that the organism is not capable of degrading tetrathionate.

The reactors were glass-made H-type two-chamber MFCs with carbon brush electrodes as both the cathode and the anode electrode (Figure 3.1). AMI-7001 anion exchange membrane (Membranes International, USA) was used to divide the chambers. MF-2052 Ag/AgCl electrode (BASi, USA) was placed in the anode compartment as a reference electrode to study the changes in the anode potential. The effective volumes of the both chambers were 250 ml; giving total effective reactor volume of 500 ml. A resistor of 1000 Ω was used as the external load.

The anolyte contained 8.8% (v/v) MSM, 17.7 mM K_2HPO_4, 1% (v/v) TES, 2.0 g/l $S_4O_6^{2-}$, 10 mM $NaHCO_3$, and 20% (v/v) inoculum in MQ water. MSM and K_2HPO_4 were sterilised in an autoclave (121 °C, 20 min), whereas TES and $S_4O_6^{2-}$ stock were filter-sterilised

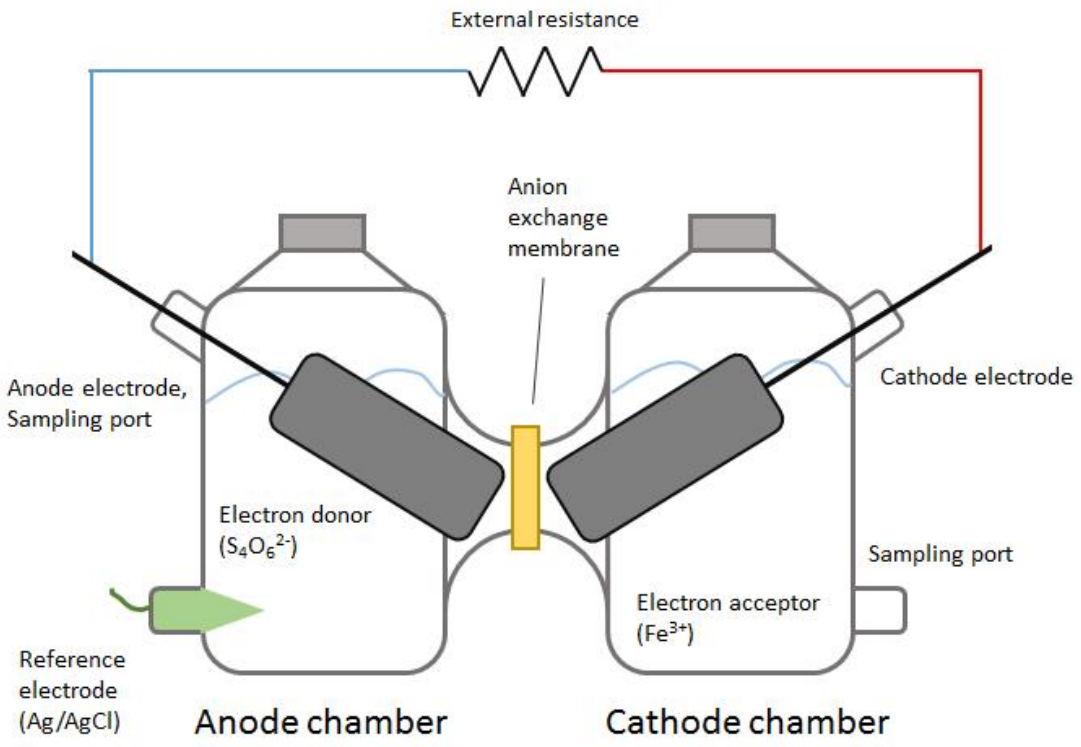

Figure 3.1 *Tetrathionate MFC setup.*

(0.2 µm cellulose acetate sterile syringe filter; VWR, USA). The MFCs were sterilised as parts in an autoclave (121 °C, 20 min) and assembled under UV-light before the addition of the solutions and the inoculum. The anolyte solution was purged with N_2 through 0.2 µm cellulose acetate sterile syringe filter (VWR, USA) for 15 minutes in the reactor bottle to dispose of any oxygen. Lastly nitrogen purged sterile $NaHCO_3$ was added to the media anaerobically to offer a carbon source prior to the inoculation. Ferric iron was used as the electron acceptor at the cathode. The catholyte consisted of 2 g/l (0.090 M) Fe^{3+}, added as $FeCl_3$, and it was sterile-filtered before the introduction to the reactor. The catholyte solution was made to MQ-water, the pH of which was set to 1.5 with HCl.

Samples (1.5 ml) were taken from the anolyte under laminar flow once a week with a syringe to avoid disturbing the anaerobic conditions inside the MFC. The samples were handled in similar manner as the 1.5 ml samples of aerobic batch bottle experiments. Nitrogen-purged sterile 1 M $NaHCO_3$ (1 ml) was added once during the experiment, at day 20, to offer a carbon source to the microbes. To re-elevate the substrate concentration back to 2.5 g/l 6 ml of sterile tetrathionate stock solution (100 g/l $S_4O_6^{2-}$) was added on day 28. LSV (0.0015 V/s scan rate) was performed with PalmSens3 potentiostat (PalmSens, Netherlands) coupled with PSTrace 4.6 software at day 32 of the experiment.

3.3 MFC start-up strategy experiment

The effect of different MFC start-up strategies was assessed with four flow-through air-cathode MFCs using simulated forest industry wastewater as the substrate. The four different start-up strategies were gradually decreasing high external resistance (5000–47 Ω; named GDR), stable low external resistance (50 Ω; SLR), high anode potential (0 mV; HAP), and low anode potential (-450 mV; LAP). The high resistance was lowered gradually to let the culture adjust to the change of the external resistance. LSV was conducted to the MFCs before any alterations to the external resistance. High initial resistance was lowered weekly and the applied resistances were 5000 Ω, 2500 Ω, 1000 Ω, 500 Ω, 100 Ω and 47 Ω. External resistance of 47 Ω was applied to all reactors during the final week of the experiment to be able to compare their performance. The period when the reactors were subjected to their corresponding conditions is referred as the start-up phase, compared to the control phase when the conditions were identical in all reactors.

The simulated wastewater (Table 3.2) was prepared as described in by Mäkinen *et al.* (2012), with exceptions that Resazurin was omitted (Mäkinen et al. 2013) and that different organic substrate was used. The wastewater was prepared by supplementing the aforementioned media solution with 2% v/v substrate stock solution and 10% v/v inoculum. The substrate stock contained 75 g/l acetate, 33.3 g/l glucose and 16.7 g/l xylose to yield the final concentrations of 1.5 g/l acetate, 0.66 g/l glucose and 0.33 g/l xylose in the MFC. The concentrations correspond to 2.5 g/l COD, with similar composition of the main components as reported for forest industry wastewaters (Pokhrel & Viraraghavan 2004).

Table 3.2 *The composition of the simulated forest industry wastewater used as the anolyte in the start-up experiment.*

Chemical	c (g/l)	Chemical	c (µg/l)	Vitamin	c (µg/l)
Acetate	1.5	$FeCl_2$	2000	Biotin	10
Glucose	0.66	H_3BO_3	50	Folic acid	10
Xylose	0.33	$ZnCl_2$	50	Pyridoxine-HCl	50
NaH_2PO_4	10.7	$CuCl_2$	38	Thiamine-HCl	25
Na_2HPO_4	3.2	$MnCl_2$	41	Riboflavin	25
NH_4Cl	0.6	$(NH_4)_6Mo_7O_{24}$	50	Nicotinic acid	25
KH_2PO_4	0.125	$AlCl_3$	50	D-Ca-pantothenate	25
$CaCl_2$	0.11	$CoCl_2$	50	Vitamin B12	0.5
$MgCl_2$	0.1	$NiCl_2$	50	p-Aminobenzoic acid	25
$NaHCO_3$	4	Na_2SeO_3	26.3	Lipoic acid	25
Na_2S	0.24	$NaWO_4$	32.9		

The effective reactor volume of the flow-through MFCs was 123 ml which was also the effective volume of the anode chamber, and the total area of the oxygen-exposed cathode electrode was 41.25 cm^2. Two carbon brushes were used as the anode electrodes and MF-2052 Ag/AgCl electrodes (BASi, USA) were used as the reference electrodes. Oxygen reduction cathode was a carbon cloth on top of CMI-7000 cation exchange membrane (Membranes International, USA). The cultivation bottles resided in a water bath at 37 °C, and the reactors itself were run in ambient temperature (20±2 °C). The reactor set-up is illustrated in Figure 3.2a. Initially 500 ml of simulated wastewater was circulated through the reactor 100 ml/min from an anaerobic bottle. As the air-cathode MFCs evaporate media through the cathode and because minor leakages and sampling reduce the media volume during the experiment, media solution (containing no substrate) was added at days 15 and 28 adjust the culture media back to the level it was in the beginning of the experiment.

The anolyte solution was kept anaerobic throughout the experiment. Anoxic conditions were achieved by purging the media and the anaerobic bottle with N_2 upon set-up, sampling and media addition. As oxygen was used as the final electron acceptor at the cathode, no oxygen can be allowed to the anode chamber. In that case the cells could use the soluble oxygen as the final electron acceptor instead of that at the cathode, resulting to non-existent cell emf.

A sample of 11.5 ml was taken from the anolyte once a week. Part (1.5 ml) of the sample was treated in similar manner as the 1.5 ml samples in aerobic tetrathionate degradation experiment. The rest (10 ml) was stored in -20°C for subsequent analyses. The samples were taken with a serological pipette directly from the anaerobic bottle. Before the sampling the bottle was purged with N_2 for at least 2 minutes to ensure that all the media solution in the reactor had been thoroughly purged to sustain the anaerobic conditions

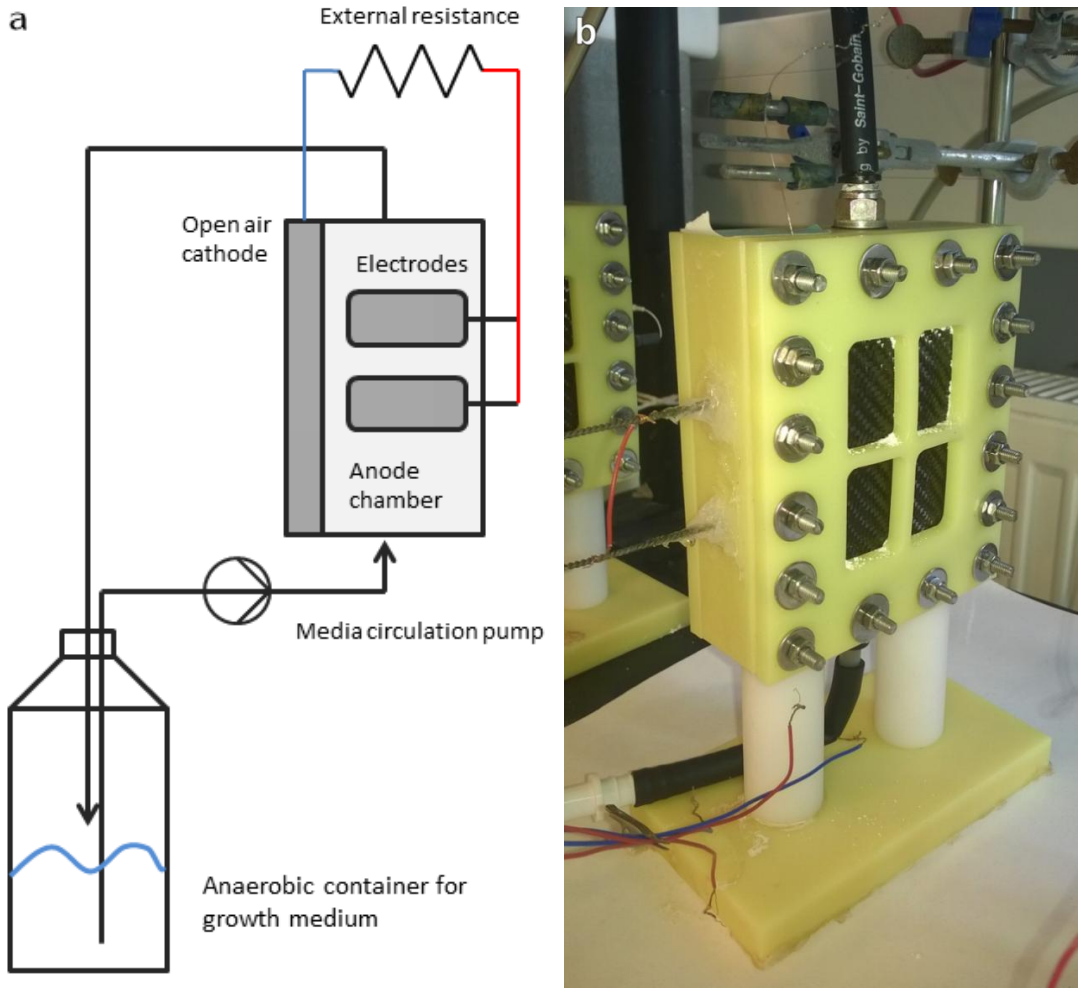

Figure 3.2 *The MFCs used in the start-up experiment.* ***a)*** *The MFC setup.* ***b)*** *Photograph of the MFC, showing the air cathode.*

inside the reactor. Media pH was measured directly from the cultivation bottle and set to 7.0–7.2 with 6 M NaOH after sampling.

LSV was performed with PalmSens3 potentiostat (PalmSens, Netherlands) coupled with PSTrace 4.6 software weekly after which the external resistance of the GDR-MFC was decreased. The anolyte pH was set to 7.0–7.2 with 6 M NaOH a day prior to the experiment and the reactors were unattached from the circuit at least 2 hours prior to commencing the LSV to ensure that the reactor had reached its OCV.

3.4 Inocula

The type strains of *Acidithiobacillus ferrooxidans* (DSM-14882; DSMZ, Germany) and *Ferroplasma acidiphilum* (DSM-12658; DSMZ, Germany) were used for tetrathionate degradation experiments. *A. ferrooxidans* strain is also known as ATCC 23270 and it was originally isolated from a bituminous coal mine effluent (Leathen & Braley 1954),

whereas the original strain of *F. acidiphilum* has been isolated from a pyrite-leaching pilot plant in Kazakhstan (Golyshina et al. 2000). The first aerobic enrichments were inoculated from aforementioned pure strains which had been cultivated in the culture media recommended by DSMZ (DSMZ 882 for *A. ferrooxidans* and DSMZ 874 for *F. acidiphilum*) and were accustomed to use ferrous iron (Fe^{2+}) as the substrate.

The tetrathionate MFCs were inoculated from the first aerobic batch experiment bottles (20% v/v) with *A. ferrooxidans* A and B, and Binary cultures A and B. The abiotic control MFC was inoculated with sterile MQ water. During the experiment more inoculant was added (10% v/v) from second (*A. ferrooxidans* B and Both B reactors) or third (*A. ferrooxidans* B and Both B reactors) aerobic enrichment batches to ensure the amount of sufficient biomass and to replenish the media volume that had dropped because of the sampling. Mixed culture from a running flow-through MFC was used as the positive control when studying tetrathionate degradation in the MFCs. The culture in the reactor had been using tetrathionate as electron donor and operated in anaerobic conditions. The culture was originally obtained from hydrometallurgical multimetal mining process water and has been enriched and re-inoculated multiple times since then.

The inoculum for the start-up experiments was received from Viinikanlahti municipal wastewater treatment plant (Tampere, Finland). The inoculum was received from the anaerobic digester of the plant one day prior to the beginning of the start-up experiment and it was stored at 4°C before the inoculation.

3.5 Analytical methods

Optical density was measured directly from the sample with UV-1700 UV-Vis spectrophotometer (Shimadzu, Japan) using 600 nm wavelength and optical glass cuvettes (Hellma Analytics, Germany) with 10 mm light path. The average value of two parallel measurements was used for each data point. All the pH measurements were done with WTW 330 pH meter (WTW, Germany) with SlimTrode electrode (Hamilton, USA).

Tetrathionate concentration was measured from the supernatant by modified cyanolysis method, described originally by Sörbo (1957) and modified by Kelly *et al*. (1969). Firstly, 300 µl of phosphate buffer (containing 0.11 M NaH_2PO_4 and 0.09 M NaOH) and 100 µl of 100 mM KCN (in H_2O) were added to 400 µl of the accordingly diluted sample containing tetrathionate. The solution was then mixed and incubated in ambient temperature (20±2 °C) in 150 rpm agitation for 60–90 minutes. After incubation, 200 µl of 1.5 M ferric nitrate in 4 M $HClO_4$ was added for colour formation. The absorbance was read at 485 nm with Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan). Acidified MQ water (pH 2.0) was used to dilute the sample and as a blank.

Sulphate and thiosulphate ion analysis was done from the filtered supernatant using Dionex ICS-1600 ion chromatograph (Dionex, USA), with Ion-Pac AS4A-SC anion exchange column, AS-DV autosampler, and ASRS-300 suppressor (2 mm). The eluent comprised 1.9 mM Na_2CO_3 and 1.7 mM $NaHCO_3$. Flow rate was set to 1 ml/min.

Ferrous iron concentration was determined with phenantroline method described by American Public Health Association (1992). Sample (1 ml) was mixed with 0.1 ml 37% HCl, 2 ml 10 g/l 1,10-phenantroline monohydrate solution (in MQ), 1 ml ammonium acetate buffer (containing 250 g/l ammonium acetate and 700 ml/l glacial acetic acid in MQ), and 0.9 ml MQ-water. The mixture was gently mixed and the absorbance read immediately at 510 nm with Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan). The blank contained 0.07 M HNO_3 which was also used to dilute the samples when needed.

COD was analysed as described in the standard SFS 5504:1998: 1.00 ml of 40.00 mM $K_2Cr_2O_7$, 3 ml 10 g/l Ag_2SO_4 in strong sulphuric acid and 2.0 ml of the sample were mixed in a closed reaction tube and heated 120 minutes at 150 °C. After the heating, the samples were cooled to ambient temperature, 1–2 drops of ferroin indicator solution was added, and the solution was titrated with 0.07 M $(NH_4)_2Fe(SO_4)$. Soluble COD (COD_s) was measured from a filtered sample (0.45 µm Acrodisc PSF Syringe Filter; Pall Life Sciences, USA), whereas total COD (COD_{tot}) was measured from an unfiltered sample. The final COD value was calculated with Equation 14 where c_{Fe} is the concentration of the iron(II) solution, $V_{Fe,blank}$ the volume of iron(II) solution used by the blank sample, $V_{Fe,sample}$ the volume of iron(II) solution used by the sample, and V_{sample} the volume of the sample.

$$COD_{Cr} = \frac{8000\, c_{Fe}\left(V_{Fe,blank} - V_{Fe,sample}\right)}{V_{sample}} \quad (14)$$

High performance liquid chromatography (HPLC) was used to analyse the concentrations of sugars (xylose, glucose), volatile fatty acids (VFAs) (lactate, formate, acetate, propionate, butyrate) and ethanol from the sample. The utilised apparatus combined Shimadzu LC-20AD liquid chromatograph, Shimadzu DGU-20A3 degasser, Shimadzu RID-10A refractive index detector and SIL-20AC HT auto sampler (Shimadzu, Japan), as well as Rezex RHM-Monosaccharide H+ LC Column (300 x 7.8 mm; Phenomenex, USA).

The voltage data and the anode potentials of each MES were recorded at 2 minute intervals with 34970A Data Acquisition/Switch Unit (Agilent Technologies, USA). The anode potentials of the anode controlled reactors in the start-up experiment were set and their current data recorded with µStat8000 Multi Potentiostat (DropSens, Spain).

4. RESULTS

Results of the study are presented one experiment at a time beginning from the experiments conducted with the simulated mining wastewater. First, the results from the aerobic degradation of tetrathionate in pure batch cultures are presented in Section 4.1. After that in Section 4.2, I present the results from tetrathionate degradation experiment conducted in MFCs with pure cultures. Lastly I produce the results from MFC start-up strategy experiments conducted with mixed microbial culture in simulated forest industry wastewater in Section 4.3.

4.1 Tetrathionate degradation in aerobic batch cultures

Tetrathionate was degraded efficiently in the aerobic cultures and growth was observed in most of the cultivations. The culture pH and the SO_4^{2-} concentration showed inverse correlation and the biomass growth (OD_{600}) correlated directly with the consumption of $S_4O_6^{2-}$ in every batch. Figure 4.1 presents all the measured parameters from the fifth enrichment as an average of the results from parallel experiments. The last (fifth) enrichment batch without the addition of extra phosphate buffer was chosen to represent the correlations of the metabolites in the culture media because of good growth and relatively short lag times observed with all the cultures.

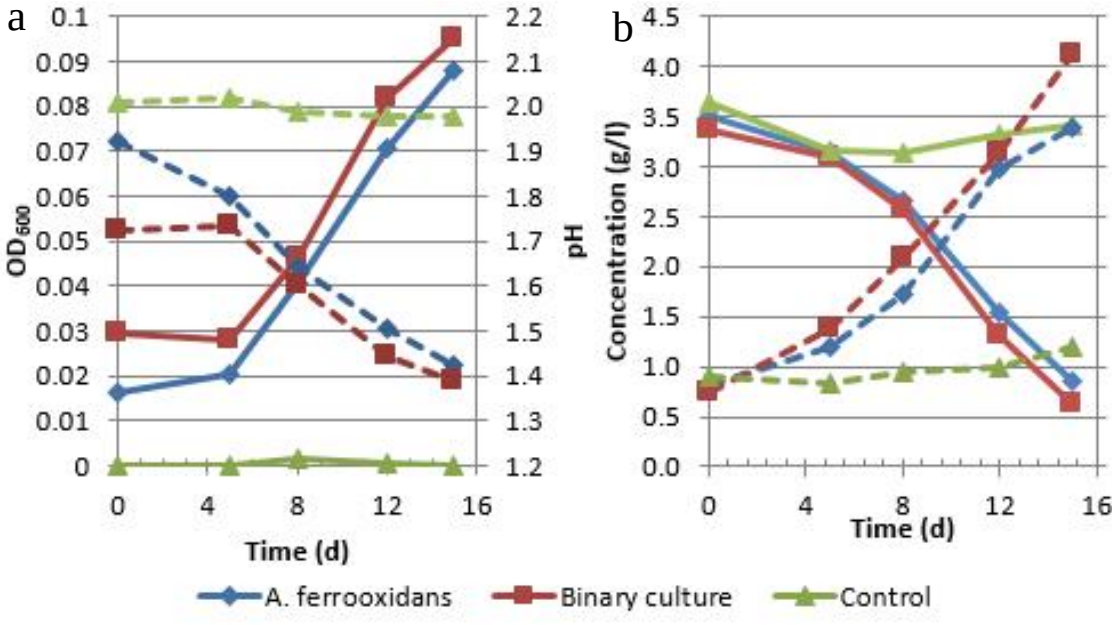

Figure 4.1 *Optical density (OD_{600}), pH, as well as SO_4^{2-} and $S_4O_6^{2-}$ concentrations as a function of time from the fifth aerobic tetrathionate degradation batch. Dashed lines are presented on the secondary axes.* ***a)*** *Optical density and pH (dashed line).* ***b)*** *Tetrathionate and sulphate (dashed line) concentrations.*

During the 15-day cultivation, tetrathionate concentrations decreased from 3.2–3.5 g/l to 0.35–1.4 g/l. Binary culture B and *A. ferrooxidans* B cultures presented a 5-day lag time before observable growth or substrate conversion which had an effect on the presented average curves. Binary and *A. ferrooxidans* cultures were able to consume tetrathionate and use it as their main energy source throughout the study (Figure 4.2). Abiotic control experiments were done parallel to the biotic batches. No $S_4O_6^{2-}$ degradation was observed under abiotic conditions in any of the batches during the study (data not shown). Neither any growth nor $S_4O_6^{2-}$ degradation was observed in cultures with *F. acidiphilum* as the sole microorganism (data not shown).

As $S_4O_6^{2-}$ started to degrade in the cultivations, pH started to decrease rapidly, biomass grew steadily, and a large amount of SO_4^{2-} was released to culture media. Total sulphur levels of the media were calculated to evaluate if elemental sulphur or other non-analysed sulphur compounds were produced in the process but no significant differences in total mass of sulphur in $S_4O_6^{2-}$ and SO_4^{2-} were observed in any of the batches. Binary cultures and the *A. ferrooxidans* cultures produced very similar cultivation patterns with all measured metabolites, binary culture being slightly faster to consume the substrate.

The cultivation times of all the batches are presented in Figure 4.2. Total cultivation time consists of the estimated lag time and the cultivation time before the start of new batches (time taken for tetrathionate concentration to decrease below 0.5 g/l), presented separately for each enrichment cultivation. Tetrathionate degradation rates were calculated as the amount of consumed $S_4O_6^{2-}$ per litre of working volume against the growth time. Tetrathionate concentration data from the batches was used to estimate the lag times of the culti-

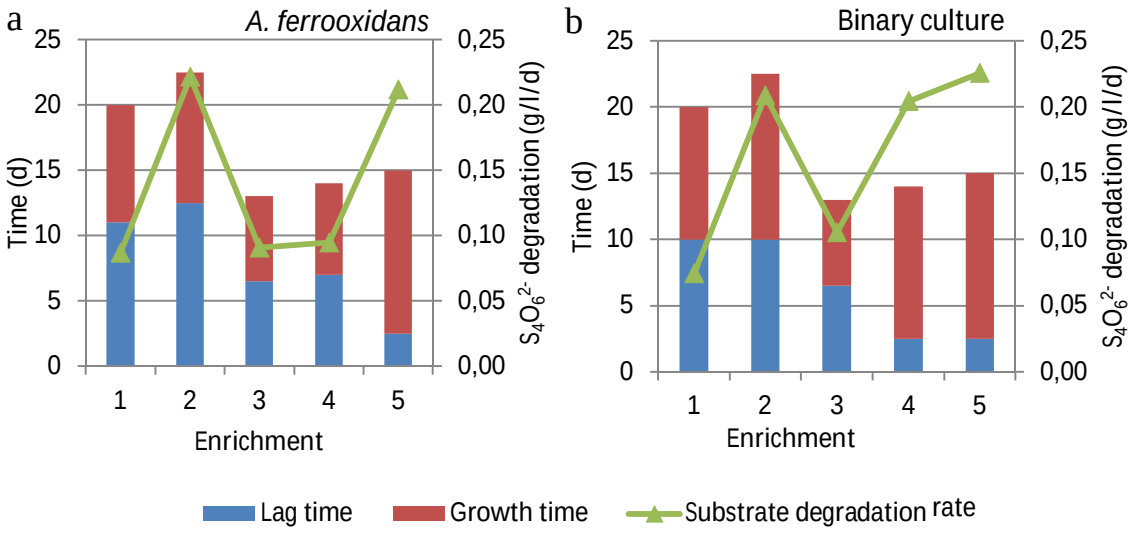

Figure 4.2 *The total cultivation times and the substrate degradation rates of aerobic tetrathionate degradation batches.* ***a)*** *The average cultivation times and substrate degradation rates of* A. ferrooxidans *cultures.* ***b)*** *The average cultivation times and substrate degradation rates of Binary cultures.*

vations. The lag time was evaluated as the time before the slope of tetrathionate concentration curve started to decrease. The lag time was evaluated as the time before any visible substrate utilisation.

The cultivations with the same cultures of *A. ferrooxidans* (A or B) produced very similar cultivation time patterns with each other (Table 4.1). The lag times were exactly the same for both A cultures but more variation can be found from the B cultures. Lag times were slightly smaller for Binary culture B cultivations than for sole *A. ferrooxidans* B cultures. Table 4.1 shows the numeric values of cultivation and lag times as well as their standard deviations for each strain separately. The cultivations that did not show any growth or substrate utilisation were not included in the calculations.

The highest average S_4O_6 degradation, 0.28 g/l/d, was observed with *A. ferrooxidans* B culture (enrichment 2). The degradation rates increased continuously from 0.17 to 0.21 g/l/d during the enrichment with *A. ferrooxidans* A cultures. The substrate was degraded steadily in all the enrichments, except the first one with average degradation rate of 0.19 g/l/d, and very short or non-existent lag times were observed. *A. ferrooxidans* B cultures showed longer lag times in the cultivations than their A counterparts and refused growth and degradation totally in some batches. However, more efficient tetrathionate degradation was observed in these cultures than in their A counterparts with average degradation rates of 0.22 g/l/d and 0.22 g/l/d with *A. ferrooxidans* B and Binary culture B, respectively. If tetrathionate was degraded, the lowest observed degradation rate was 0.15 g/l/d (Binary culture B, enrichment step 1).

As the $S_4O_6^{2-}$ degradation produces large amounts of protons, which decreases the pH, it was tested if low pH was inhibiting the organisms and if it could be controlled with additional buffer. This was tested with addition of phosphate buffer (final concentration 100 mM PO_4^{3-}) to the growth media. Along with the fifth enrichment two buffered cultivations were inoculated from fourth round Binary culture cultivations to see if any changes occur after phosphate addition. The $S_4O_6^{2-}$ degradation and the pH curves of the buffered cultivations and their non-buffered counterparts are presented in Figure 4.3. The addition of

Table 4.1 *Average cultivation times and tetrathionate degradation rates in aerobic batch cultivations presented for each culture separately. Data was gathered from 5 separate enrichment batches. σ, standard deviation.*

	At. ferrooxidans A		*At. ferrooxidans* B		Binary culture A		Binary culture B	
	Average	σ	Average	σ	Average	σ	Average	σ
Cultivation time (d)	14.5	1.3	21.3	7.1	14.5	1.3	19.5	6.9
Lag time (d)	0.5	1.0	10.0	11.4	0.5	1.0	7.0	7.7
$S_4O_6^{2-}$ degradation rate (g/l/d)	0.19	0.020	0.22	0.052	0.19	0.019	0.22	0.051

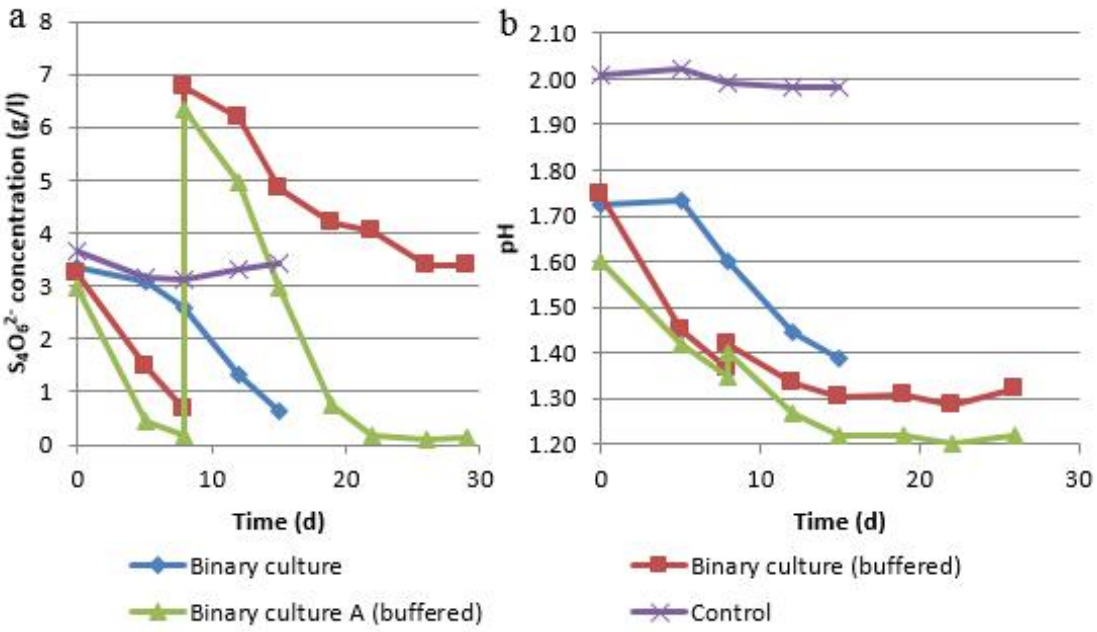

Figure 4.3 a) *The tetrathionate degradation by the Binary cultures in non-buffered and* PO_4^{3-} *buffered growth media and by Binary culture A in the buffered medium during the fifth enrichment.* ***b)*** *The change in the medium pH during the non-buffered and* PO_4^{3-} *buffered cultivations by Both A and Both B cultures during the fifth enrichment.*

the buffer speeded up the degradation, and all $S_4O_6^{2-}$ had been degraded after eight cultivation days in Buffered A culture. Both buffered cultures degraded $S_4O_6^{2-}$ faster than the corresponding culture without added phosphate. More $S_4O_6^{2-}$ was then added to the buffered cultivations (double the initial substrate concentration) to see if the culture is able to cope with rising amounts of tetrathionate. However, after addition of extra substrate only culture A was able to degrade tetrathionate. Medium pH dropped rapidly in all cultivations, despite the buffering.

As the declination of tetrathionate degradation rate in the Buffered B culture after $S_4O_6^{2-}$ addition was thought to arise from low pH (1.40), one batch experiment was done with buffered growth medium. Tetrathionate degradation and alterations in the media pH are presented in Figure 4.4. Tetrathionate degradation was slow in the sixth batch enrichment. After 19 days, as the experiment was discontinued, the most efficient culture had achieved 43% degradation of initial tetrathionate. The lowest observed pH was 1.77.

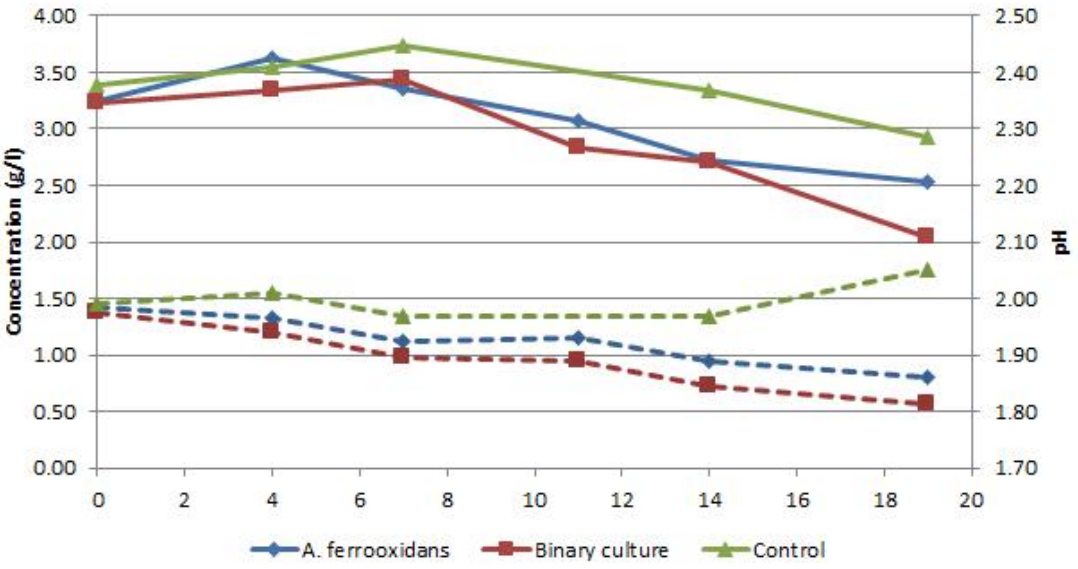

Figure 4.4 *The tetrathionate concentration and the pH (dashed line) curves in the PO_4^{3-} buffered growth media.*

4.2 Coupling tetrathionate degradation and electricity generation in MFCs

Tetrathionate degradation was studied in four experimental MFCs and one abiotic control MFC. During the 72-day experiment the highest obtained cell voltages were 3.2 mV, 3.5 mV, 1.8 mV, 2.0 mV, and 1.7 mV for *A. ferrooxidans* A, *A. ferrooxidans* B, Binary culture A, Binary culture B, and for abiotic control MFCs, respectively (Figure 4.5). Maximum power densities were calculated from the maximum voltages and were

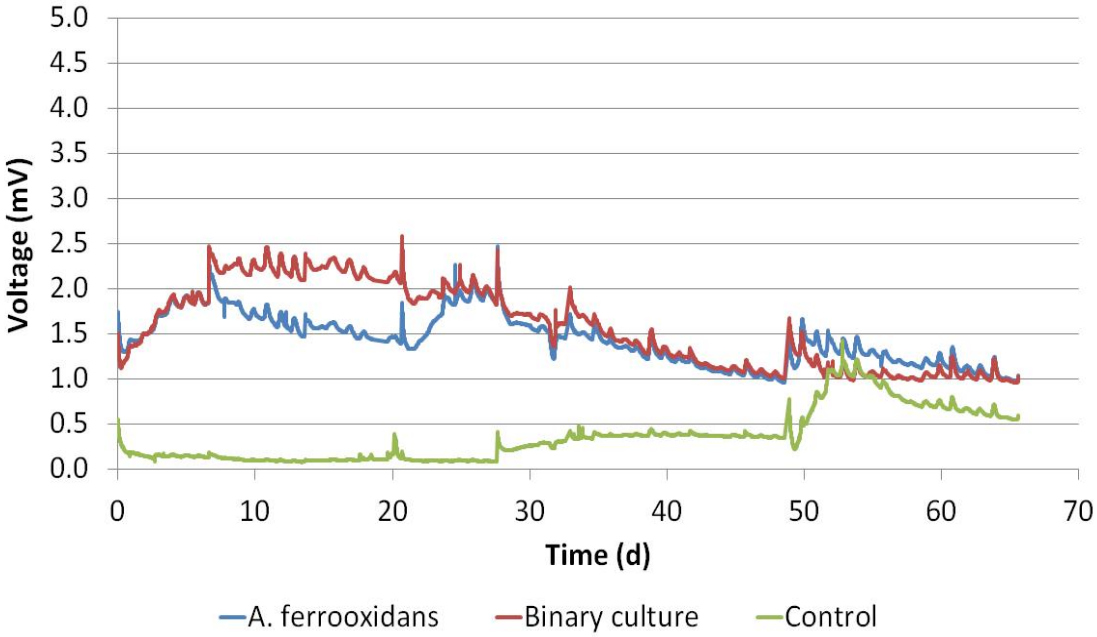

Figure 4.5 *The average cell voltages of the tetrathionate degrading cultures in MFCs during the 72-day experiment.*

0.04 mW/m^3, 0.05 mW/m^3, 0.01 mW/m^3, 0.02 mW/m^3, and 0.01 mW/m^3 for *A. ferrooxidans* A, *A. ferrooxidans* B, Binary culture A, Binary culture B, and for abiotic control MFCs, respectively.

The performance of the pure culture MFCs was further compared to a biotic control MFC to validate that the experimental setting was not limiting the current production. The biotic control MFC contained mixed microbial culture which had been enriched multiple rounds in a flow-through MFC using tetrathionate as the main substrate. For this, the *A. ferrooxidans* A MFC was discontinued and replaced with the biotic control MFC. The biotic control MFC reached 16 mV maximum voltage and 1.0 mW/m^3 maximum power density which are 4.5 and 20 times higher than those obtained with pure cultures (Figure 4.6).

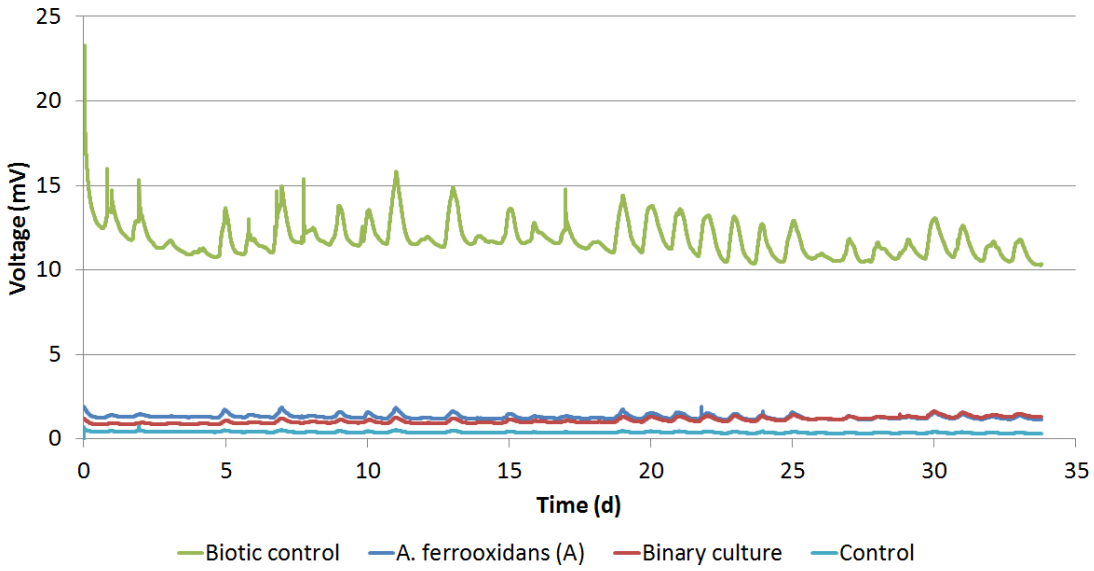

Figure 4.6 *The voltage curve of the biotic control MFC compared to the other tetrathionate degrading MFCs.*

During the cultivation the tetrathionate concentration decreased in all MFCs, as presented in Figure 4.7a. On day 28, more $S_4O_6^{2-}$ was added as the concentration reduced below 0.5 g/l. This did not affect the observed voltages but the consumption of $S_4O_6^{2-}$ slowed down after the tetrathionate addition. The SO_4^{2-} concentration remained stable throughout the experiment (Figure 4.7b) which suggests that tetrathionate was not degraded. Most likely the decrease in $S_4O_6^{2-}$ concentration was caused by the migration of the ions to the cathode chamber. The rapid drop and as rapid return in the SO_4^{2-} concentrations at day 21 in all biotic cells is caused most probably by poor sampling. The anolyte pH decreased steadily to 1.5 in all MFCs after which it was adjusted back to 2.0–2.75 (Figure 4.7b). The raise of the pH did not, however, have any effect on the degradation of $S_4O_6^{2-}$ nor the produced voltage. The concentration of Fe^{3+} in the catholyte did not reduce significantly during the experiment, from 1.5 g/l to 1.3 g/l on average.

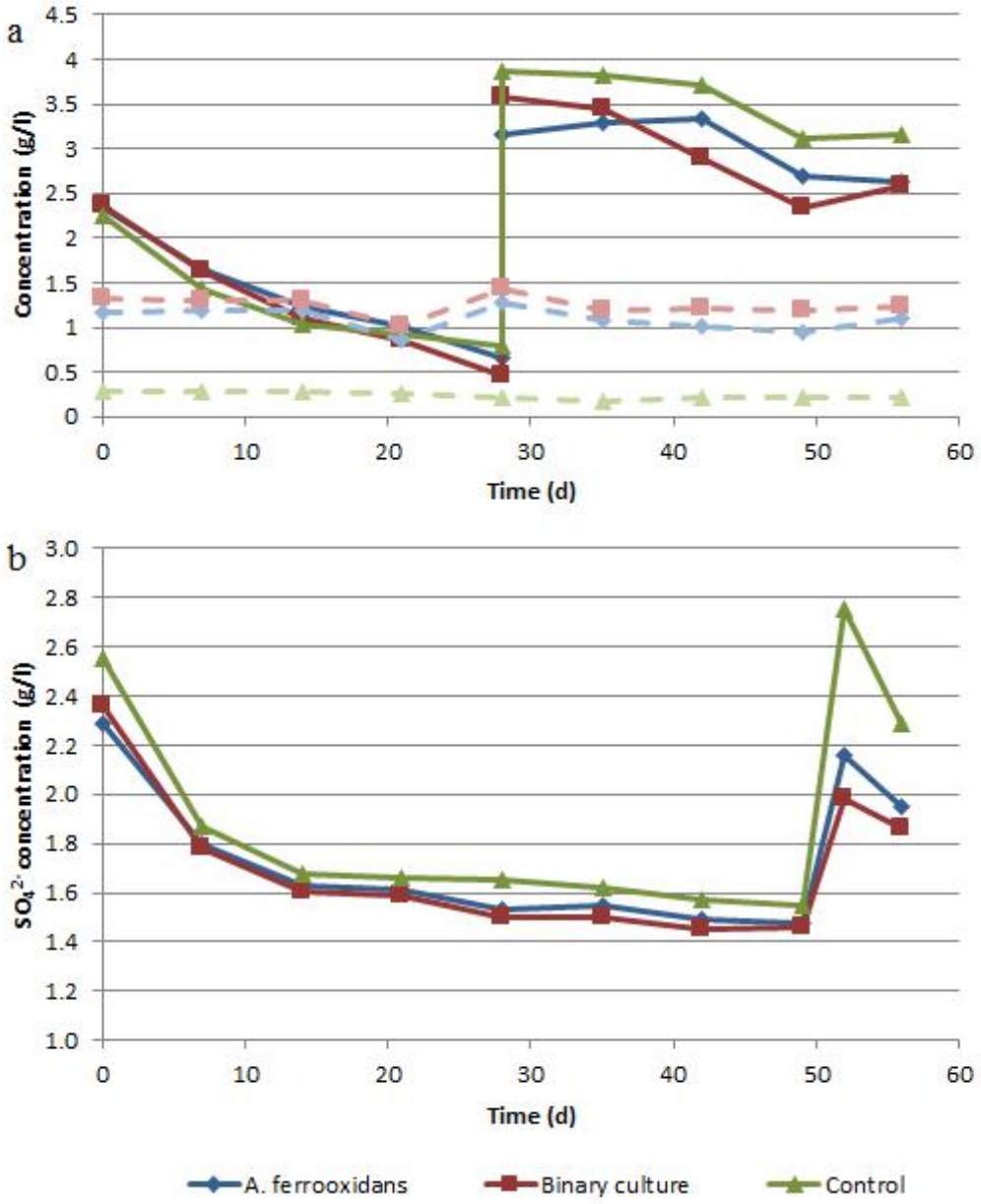

Figure 4.7 a) *The concentrations of tetrathionate and sulphate (dashed line) in tetrathionate degrading MFCs.* ***b)*** *The anolyte pH of the tetrathionate degrading MFCs. The drop of SO_4^{2-} concentration on day 21 is likely caused by non-representative sampling.*

According to the polarisation curves of the tetrathionate degrading MFCs (Figure 4.8), the maximal power densities of the reactors were on average 1.6 mW/m^3 and 1.8 mW/m^3 for *A. ferrooxidans* and Binary culture MFCs and 0.9 mW/m^3 for the abiotic control MFC, respectively. Polarisation curves clearly indicate that the inoculated MFCs did not have significantly more activity than the abiotic control MFC. Internal resistances of the MFCs were calculated from the polarisation curve as the slope of the voltage curve (Equation 12) and were for *A. ferrooxidans* and Binary culture MFCs on average 39.2 kΩ and 39.6 kΩ, respectively.

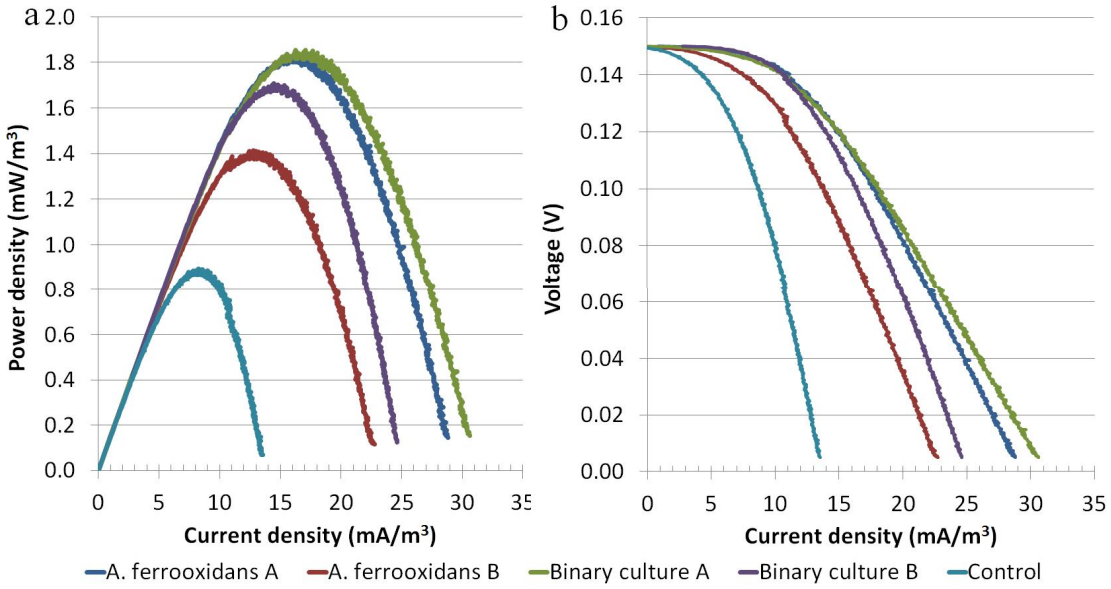

Figure 4.8 *The power density and the polarization curves of the tetrathionate degrading MFCs and the abiotic control MFC on day 32 of the experiment.* ***a)*** *Power density curves of the tetrathionate degrading MFCs and the abiotic control MFC.* ***b)*** *The polarisation curves of the tetrathionate degrading MFCs and the abiotic control MFC.*

4.3 MFC start-up experiments

The results of the start-up experiments are presented in three different parts. In Section 4.3.1 I present the electricity generation in the MFCs. I expound how the substrate was utilised and metabolised produced in the MFCs in Section 4.3.2. And finally, in the Section 4.3.3, I present the results of the performance analyses conducted to the MFCs.

4.3.1 Electricity generation in the cells

The start-up took 38 days in total after which the MFC performance was monitored in control phase for 10 days. After the control phase (day 48), the anolyte solution of the MESs was changed completely for process performance tests. The fresh media solution (roughly 600 ml) was pumped through the reactors after which the anaerobic recirculation bottle was replaced with 500 ml of fresh nitrogen-purged media solution supplemented with 2% (v/v) substrate stock solution.

In MFCs that were connected to the external resistors, anodic potentials reduced quickly, in roughly 5 days, to -438 mV (GDR) and -418 mV (SLR) which indicates that the microbial consortia had adapted to the new environment (Figure 4.9b). The highest observed cell voltages during the start-up period (Figure 4.9a) obtained with SLR and GDR were 93 mV and 530 mV (5000 Ω), and the lowest obtained anode potentials were -487 mV and -486 mV against Ag/AgCl electrode, respectively. GDR produced the highest voltage with high external resistance, but high voltage was also observed with lower external

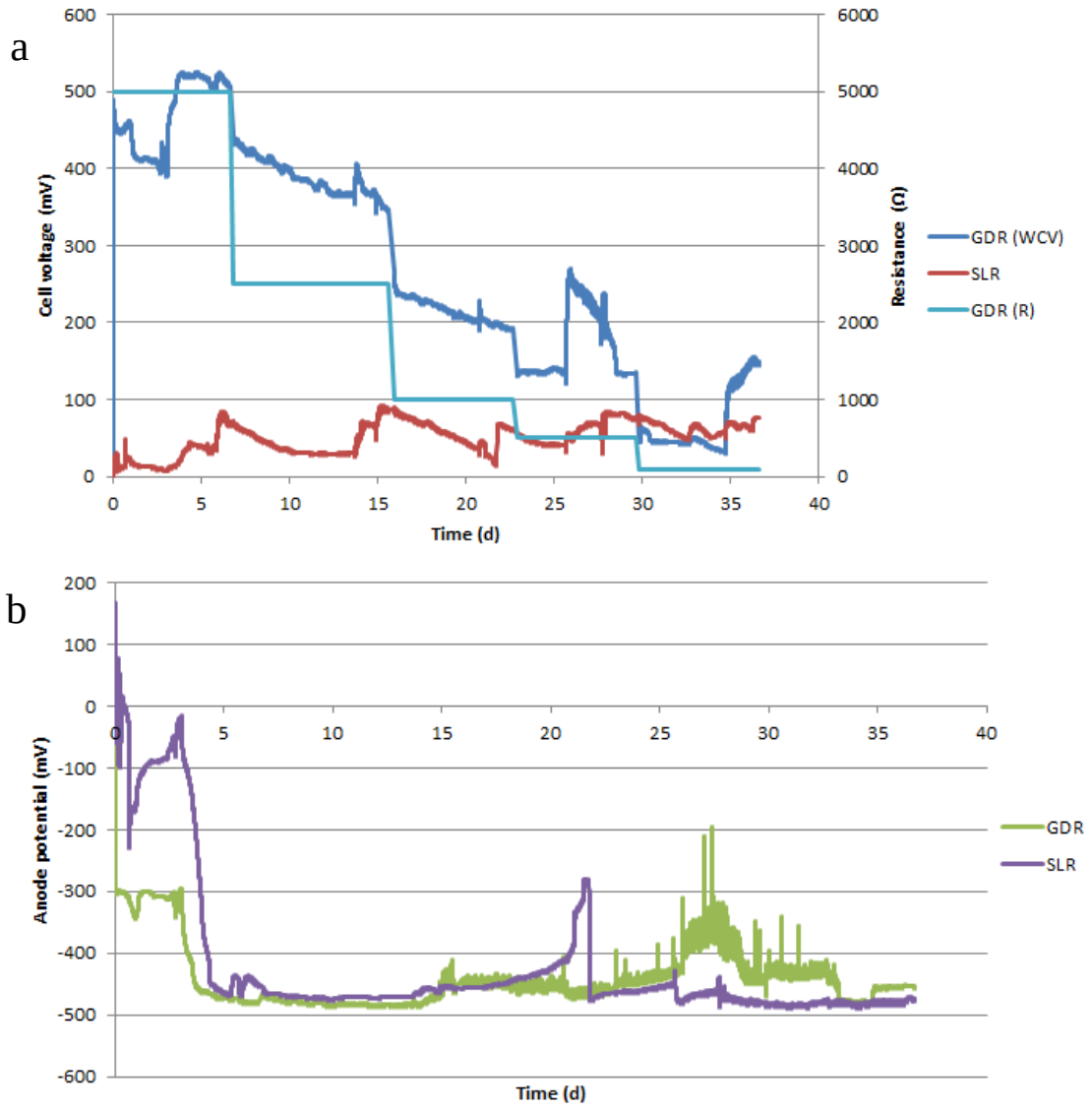

Figure 4.9 a) *The voltage generation and the applied external resistance in GDR and SLR MFCs during the start-up.* ***b)*** *The anodic potentials of GDR and SLR during the start-up. WCV, whole cell voltage; R, resistance.*

resistances (500 Ω and 100 Ω). The voltage generated in SLR varied between 30 mV and 85 mV, with an average voltage of 52 mV.

The HAP produced an extremely high peak of current production on days 6–13 with absolute maximum current on day 7. Little to no current was observed in the LAP throughout the experiment (Figure 4.10). The highest current productions observed in the HAP and LAP MESs were 73 A/m^3 and 7.6 A/m^3 and the average current productions were 13.8 A/m^3 and 0.2 A/m^3, respectively. Negative current was observed in the LAP during some days in the end of the experiment which partly explains the notably low average production in the MES. The electricity generation during the start-up phase of the MESs is presented numerically in Table 4.2.

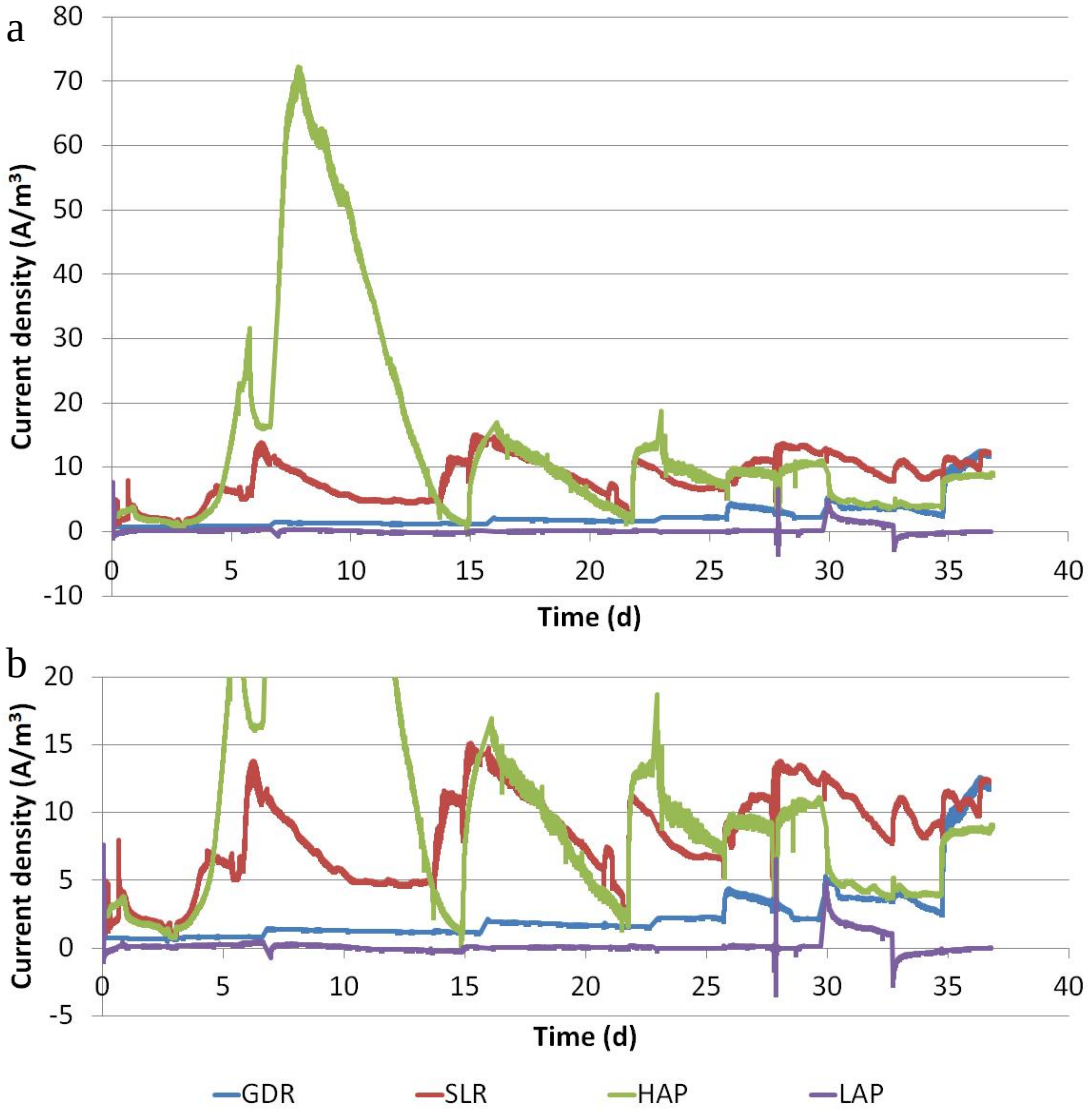

Figure 4.10 a) *The current densities during the start-up phase.* ***b)*** *A more detailed view of the current densities during the start-up of the MFCs.*

High current densities compared to the other reactors were obtained from two MESs, HAP and SLR, in the beginning of the start-up (Figure 4.10). High current generation was observed in GDR as the external resistance was decreased. The most effective period of the highest current producing MES, HAP, lasted 7 days which suggests that the high production did not result from system malfunction. The troubleshooting of the reactor's waning performance showed that the drop of the anolyte pH reduced electricity yields which led to an alteration in pH adjustment frequency. Anolyte pH was in the beginning of the experiment followed only upon sampling but the routine was subsequently (after day 22) changed so that the anolyte pH was measured and set to 7.0 ± 0.2 thrice a week in all MESs.

Table 4.2 *The electricity generation during the start-up phase of the MFCs.*

	Reactor setting			
	GDR	SLR	HAP	LAP
V_{max} (mV)	526	93	N.R.	N.R.
P_{max} (mW)	0.24	0.17	N.R.	N.R.
$I_{v,\,max}$ (A/m^3)	18	15	72	12
$I_{v,\,avg}$ (A/m^3)	3.5	8.4	13.8	0.2
AP_{min} (mV)	-486	-487	N.R.	N.R.
AP_{avg} (mV)	-439	-422	N.R.	N.R.

Abbreviations: N.R., not recorded; R_{ext}, external resistance; V, cell potential; P, power; I_v, current density; AP, anode potential

After the start-up phase, all the reactors were subjected to 47 Ω external resistance to be able to compare the performance of the MFCs in similar conditions. In the beginning of the control period, high current density (17.5 A/m^3) was obtained from GDR but its observed current production decreased throughout the start-up period, being only 9.6 A/m^3 in the end (Figure 4.11). Increasing current densities from 8.1 A/m^3 and 12.9 A/m^3 to 15.2 A/m^3 and 14.0 A/m^3 were recorded for the HAP and the SLR systems, respectively. The current production abated in the SLR for the first two days of the control period, ameliorated generating an oscillating pattern, and then increased steadily for last four days of the period. It is likely that the oscillating pattern was caused by a drop in the medium pH and the following adjustment. The LAP produced only low current (0.9–1.4 A/m^3). The average current densities during the control period were 13.2 A/m^3,

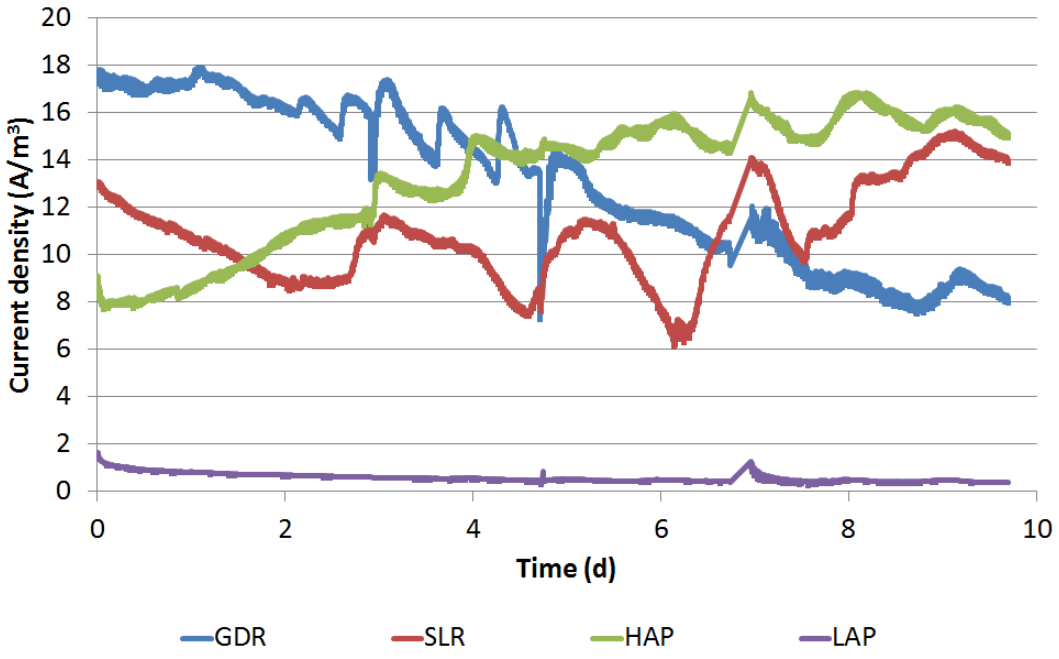

Figure 4.11 *The current densities of the start-up MESs during the control period at 47 Ω external resistance.*

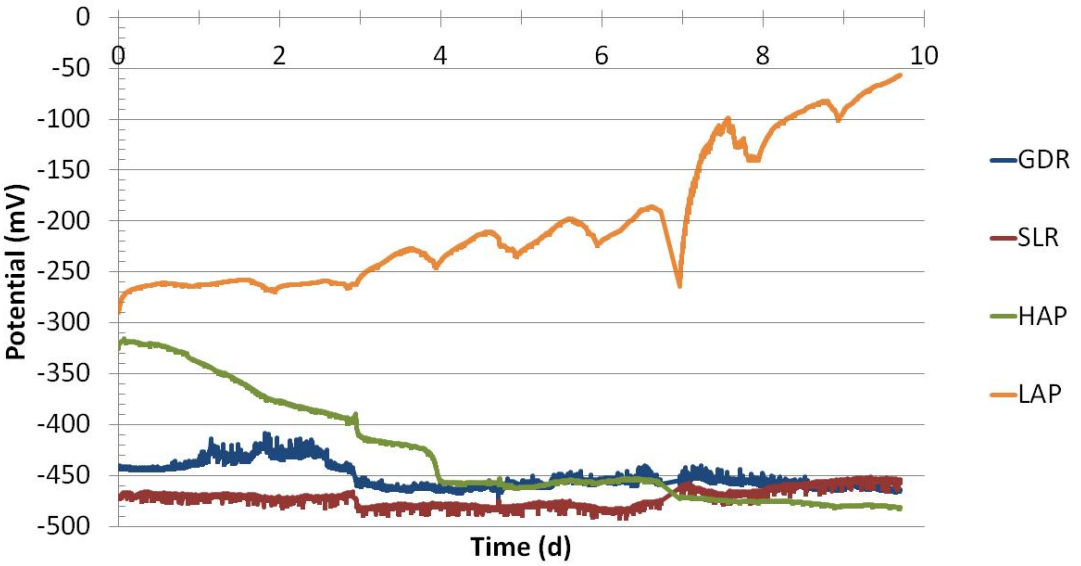

Figure 4.12 *The anodic potentials of the start-up MFCs after subjecting all the reactors to 47 Ω external resistance.*

10.9 A/m^3, 13.3 A/m^3, and 0.6 A/m^3 in GDR, SLR, HAP, and LAP, respectively. Maximum and average voltages obtained during the control period were 104 mV, 88 mV, 98 mV and 9.6 mV, as well as 76 mV, 63 mV, 77 mV, and 3.4 mV in GDR, SLR, HAP and LAP, respectively (Figure 4.12). The lowest observed anode potentials from the reactors in the same period were -472 mV, -491 mV, s-482 mV, and -290 mV, respectively.

4.3.2 Metabolite conversion

Soluble COD of the anolyte was measured weekly. Surprisingly, the COD_s began to accumulate to the anolyte instead of being utilised as the substrate (Figure 4.13). As the

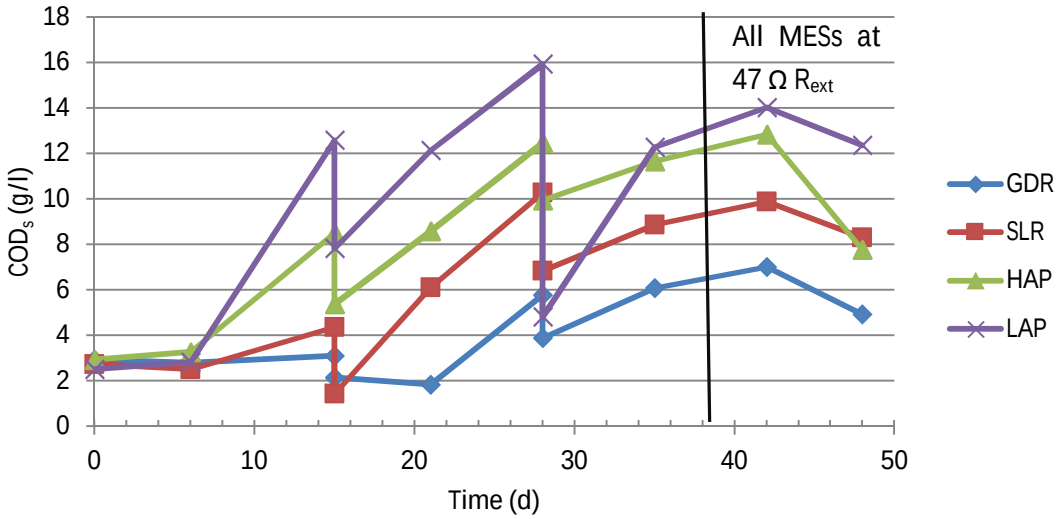

Figure 4.13 *The chemical oxygen demand of the MESs during the start-up. The drops in COD (days 15 and 28) are caused by the addition of fresh media to the MESs.*

COD levels rose throughout the start-up phase of the experiment, the MESs were not fed during the study. The MESs were started with 2.5–2.9 g/l COD_s. The maximum observed soluble CODs were 7.0 g/l, 10.0 g/l, 12.9 g/l, and 15.9 g/l in GDR, SLR, HAP, and LAP, respectively. When all the MESs were subjected to the 47 Ω external resistance, the COD_s was degraded in every MES. The highest degradation efficiency was observed in HAP (1.6 g/l COD_s, 39.5%). The COD_s degradation efficiencies during the control stage of the experiment (days 42–48, or 4–10 of the control stage) were 29.8%, 16.0%, and 11.9% for GDR, SLR, HAP, and LAP, respectively.

VFA analysis of the anolyte during the start-up revealed that various volatile acids were produced of which acetate, propionate and butyrate were dominant (Figure 4.14). Ethanol was also detected in multiple samples in rather small concentrations. The acetate levels rose in all MESs except the GDR. The highest concentrations of acetate, propionate and butyrate were 4.5 g/l, 0.9 g/l, and 220 mg/l; 10.3 g/l, 1.6 g/l, and 410 mg/l; 13.5 g/l, 2.7 g/l, and 250 mg/l; as well as 19.6 g/l, 3.1 g/l, and 320 mg/l in GDR, SLR, HAP, and LAP, respectively. Propionate levels exhibited the most stable increase, whereas acetate and butyrate were occasionally degraded. Initial concentrations of glucose (660–790 mg/l) and xylose (370–380 mg/l) were degraded in all reactors within a week (data not shown). In addition, an unidentified compound with slightly lower retention time in HPLC than that of glucose was extremely abundant in all the samples.

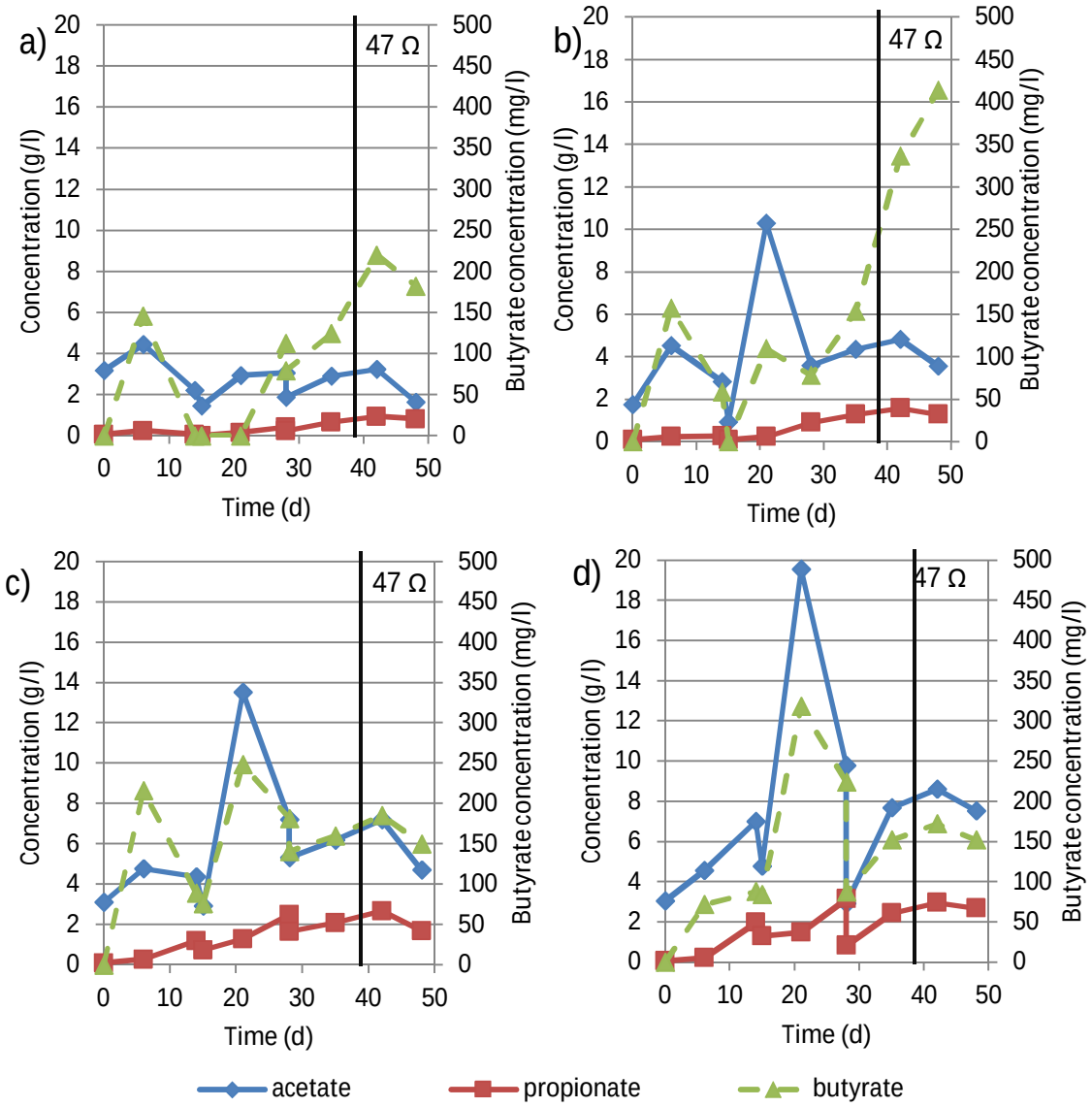

Figure 4.14 *The volatile fatty acid concentrations during the start-up experiment.* ***a)*** *GDR.* ***b)*** *SLR.* ***c)*** *HAP.* ***d)*** *LAP. The rapid decline of the concentrations on days 15 and 28 are caused partly by the addition of media. The butyrate concentrations are presented at the secondary vertical axes.*

As notable amounts of COD_s was produced to the medium, it was assumed that organic matter that is not shown as COD_s was added to the reactors with the inoculum. The metabolite profiles were measured with HPLC directly after the anolyte change (day 48) and after one-week operation. The sugars were degraded efficiently in the MFCs generating VFAs in return (Figure 4.15). The acetate was degraded in GDR but generated in other MFCs. The COD_s levels advanced during the same period from 3.0 g/l to 3.4 g/l, 3.3 g/l to 3.5 g/l, 3.3 g/l to 4.8 g/l, and from 3.5 g/l to 5.6 g/l in GDR, SLR, HAP, and LAP, respectively.

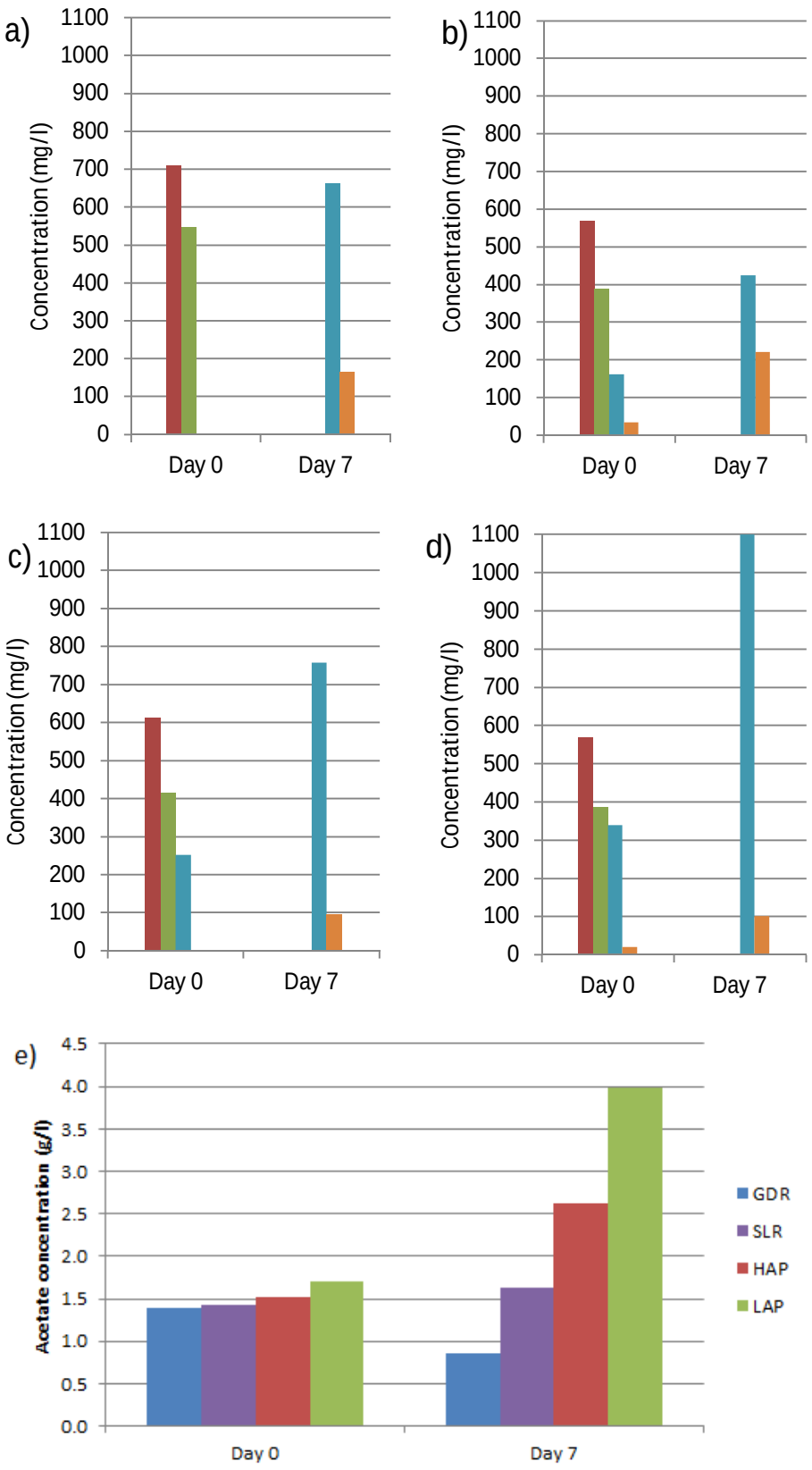

Figure 4.15 *Volatile fatty acid concentration changes during one week run after total media change (days 48–55).* ***a)*** *GDR,* ***b)*** *SLR,* ***c)*** *HAP,* ***d)*** *LAP.* ***e)*** *Acetate concentration in all start-up MFCs.*

The Coulombic efficiencies during the whole experiment (days 0–47) were calculated with Equation 10 and were 17.0%, 28.0%, 40.4%, and 0.7% for GDR, SLR, HAP, and LAP, respectively. The initial concentrations of the substrates were used with presumption that all the fed acetate, glucose and xylose was used for electricity generation. If the measured changes in acetate concentrations were used, the Coulombic efficiencies were negative (-1.3% to -3.8%), due to the accumulation of the acetate to the MESs.

4.3.3 Electrochemical characterisation of the MES performance

The performance of the MESs was monitored with weekly LSV analyses always before decreasing the external resistance in the GDR. The final analyses, performed at the end of the experiment (day 48, Figure 4.16), show that two MESs (HAP and SLR) produced significantly higher power densities compared to the other two MESs. The performance analysis results varied during the start-up (Table 4.3). The reactors were subjected to 47 Ω external resistance after the LSV conducted on the day 37.

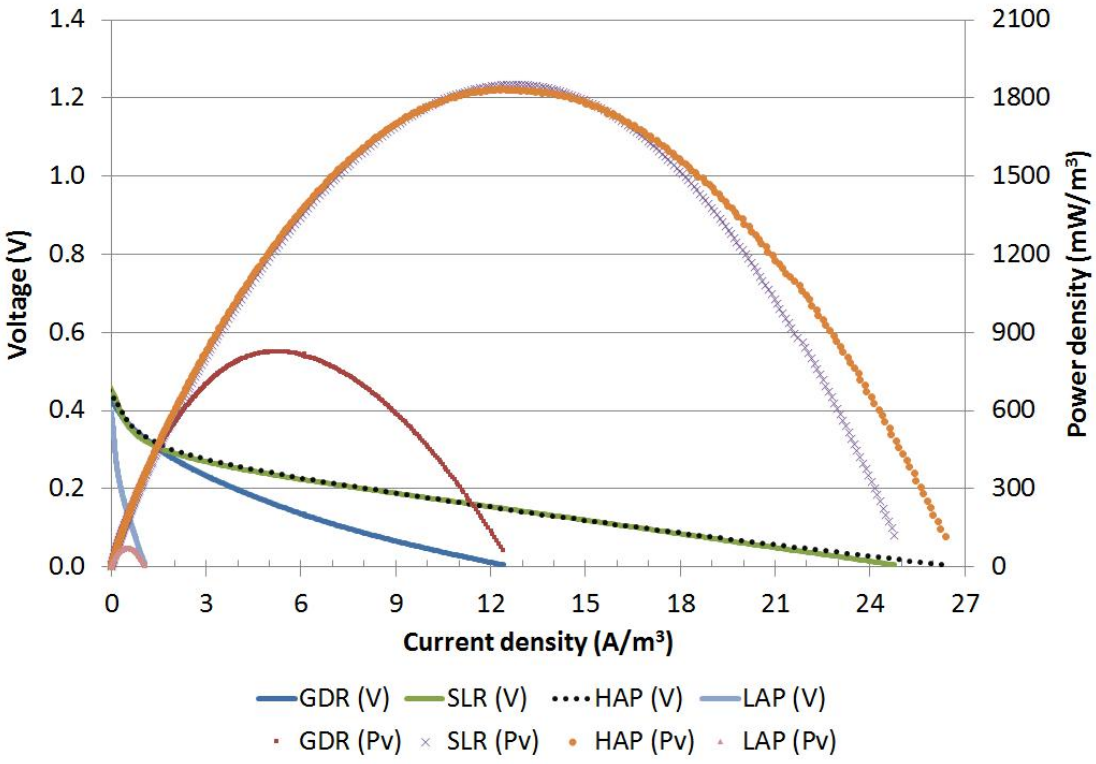

Figure 4.16 *Performance analysis, day 48. V, voltage; Pv, power density.*

The highest maximal power density (2330 mW/m^3) was produced by the GDR just before all the MESs were subjected to the 47 Ω external resistance. However, the highest average maximal power density (1320 mW/m^3) was acquired from the SLR. High maximal power densities were observed from the GDR and the SLR throughout the start-up. As all the MESs were subjected to the same external resistance, the HAP produced high power, whereas the power generation in the GDR decreased. It is also noteworthy that the HAP

Table 4.3 *Internal resistances, maximum power densities and open circuit voltages of the MFCs during the start-up and control phases.*

Time (d)	Reactor	7	16	23	30	37	47[a]
Internal resistance (Ω)	GDR	840	483	633	646	77	203
	SLR	177	14	160	131	120	100
	HAP	137	178	229	212	133	94
	LAP	564	546	388	831	761	2876
Max power density (mW/m^3)	GDR	1016	583	428	378	2330	826
	SLR	1234	1544	845	1241	1216	1852
	HAP	834	243	165	223	751	1835
	LAP	636	715	663	509	214	71
OCV (mV)	GDR	710	650	570	543	513	403
	SLR	495	510	410	420	403	439
	HAP	380	193	152	228	314	426
	LAP	790	793	752	737	804	366

[a] After all the reactors had been operated with 47 Ω external resistance for 10 days.

produced low power output during the start-up, but high output after 10-day operation attached to external resistor.

The highest OCV (804 mV) was detected in the LAP until day 47. The OCV decreased in stable manner in the GDR. During the start-up, the HAP produced high OCV which started to decrease towards the end of start-up phase and ended the control week in almost the same OCV as the GDR and the SLR.

5. DISCUSSION

The results of the tetrathionate degradation experiments in the batch bottles and the MFCs are discussed first, in Section 5.1. After that I proceed to discuss the results found in the MFC start-up strategy experiments, carried out in air-cathode MFCs using mixed microbial culture and forest industry wastewater. Suggestions for future work are given in the end of each section.

5.1 Electricity generation from tetrathionate

Tetrathionate was readily degraded in the aerobic batch bottle cultivations but the performance was not capitalised in the MFCs. The reasons to the low electricity gain from the reactors are discussed in this section and propositions are made regarding the future experiments.

5.1.1 *A. ferrooxidans* is the effective organism in aerobic cultivations

The growth times (the time organisms spent consuming tetrathionate) and the tetrathionate degradation rates (Table 4.1) strongly suggest that *A. ferrooxidans* is the organism responsible for tetrathionate oxidation observed in this study. This is supported also by various literature sources (Golyshina et al. 2000; Kelly & Wood 2000; Valdés et al. 2008; Quatrini et al. 2009; Sulonen et al. 2015; Sulonen et al. 2016), according to which *F. acidiphilum* is not capable of growth on reduced sulphur compounds whereas *A. ferrooxidans* is. Moreover, it was noticed that in the aerobic cultivations all tetrathionate was oxidised to sulphate which in addition to acidity was produced as the main outcome of tetrathionate degradation. Furthermore, the cultures were successfully accustomed to utilising tetrathionate as their energy source in aerobic conditions.

Except for the trace amounts of yeast extract, the medium in the aerobic degradation batches contained no organic sources of carbon which could support the growth of heterotrophic *F. acidiphilum*. If the organism grew in this environment, as presented previously by Sulonen (2013), it could most likely utilise small organic compounds which *A. ferrooxidans* excretes to the medium or oxidise ferrous iron. However, the existence of *F. acidiphilum* in the binary cultures was not confirmed by e.g. denaturating gradient gel electrophoresis or real-time PCR. Thus, it is possible that the organism had been eradicated from the aerobic binary cultures or from the MFC as had been found in a long-term study with these organisms (Sulonen et al. 2016). As the MFCs were inoculated with the aerobic batch cultivations, the existence and the survival of the organism in the binary culture MFCs is not certain.

The experiments with more rigorously buffered solutions of aerobic cultivations did not provide any significant benefit to maintain higher culture pH. Because the aerobic oxidation of a mole of tetrathionate produces six moles of protons (Eccleston & Kelly 1978), the pH drops rapidly as the substrate begins to be consumed (Figure 4.3). Tetrathionate was degraded from the culture media even in culture pH of 1.20, even though pH lower than 1.3 should be inhibitive to *A. ferrooxidans* (Kelly & Wood 2000). This might be caused by living cells in the culture or by extracellular tetrathionate degrading TetH protein (Kanao et al. 2007; Beard et al. 2011) that may reside in the medium even if the microbial growth was inhibited. The medium pH declined towards 1.5 in the MFC experiments which cannot be attributed to the microbial degradation of tetrathionate as the tetrathionate levels did not reduce more than in the abiotic control MFC. It is more likely that the protons were able to diffuse through the anion exchange membrane from more acidic (pH 1.5) cathode by e.g. forming different hydrogen phosphates that are able to pass the anode exchange membrane, producing the observed acidification.

5.1.2 Low biomass hampered the performance of the MFCs

Tetrathionate is oxidised biologically via hydrolysis in various microorganisms, including *A. ferrooxidans*. In the reaction, tetrathionate breaks down to elemental sulphur, thiosulphate, and sulphate, drawing an oxygen from a water molecule in the process as presented in Equation 15. This is supported by earlier findings that indicate that elemental sulphur is produced in oxygen deprived (Beard et al. 2011) as well as oxygen rich (Meulenberg et al. 1993) conditions by *A. ferrooxidans*. The thiosulphate produced in the process can be further oxidised to sulphate to yield free electrons and acidity as presented in Equation 16 (Sulonen et al. 2015).

$$S_4O_6^{2-} + H_2O \rightarrow S^0 + S_2O_3^{2-} + SO_4^{-} + 2\,H^+ \quad (15)$$

$$S_4O_6^{2-} + 6\,H_2O \rightarrow S^0 + 3\,SO_4^{2-} + 12\,H^+ + 8\,e^- \quad (16)$$

The theoretical maximum voltage that a MFC in which tetrathionate is degraded as presented in Equation 16 with ferric iron reducing cathode and in the conditions used in this experiment can produce is 714 mV (Table 5.1). The maximum voltages attained were 3.53 mV from *A. ferrooxidans* B containing MFC and 15.8 mV from the biotic control MFC. The results are far from the theoretical maxima (0.5% and 2.2%) even with the biotic control culture which is known to produce more voltage in similar settings. From the miniscule obtained voltages and the observed high internal resistances can be concluded that the MFC itself was not functioning optimally. Moreover, previously Sulonen *et al.* (2015) were able to produce 0.93 W/m^3 power density in a similar experimental setting as in this study, and Zhao *et al.* (2009) generated 149 W/m^3 power density from reduced sulphur compounds in neutral pH which are both several magnitudes higher results than the 0.050 mW/m^3 produced in this study.

Table 5.1 *The theoretical and the condition-specific reduction potentials for tetrathionate consuming MFCs. The Gibbs' free energy values were obtained from Thauer* et al. *(1977).*

Reaction	E° (V)	E vs. NHE (V)	E vs. Ag/AgCl (V)
$S_4O_6^{2-} + 6\ H_20 \rightarrow S_0 + 3\ SO_4^{2-} + 12\ H^+ + 8\ e^-$	0.274 [a]	0.056 [b]	-0.144
$Fe^{3+} + e^- \rightarrow Fe^{2+}$	0.770	0.770 [b]	0.570
Cell potential (E_{emf})[c]	0.496	0.714	0.714

[a] Calculated with Equation 5, [b] Equation 8, [c] Equation 9

The conditions used in the calculations: pH = 2.0, T = 20 °C,
$[S_4O_6^{2-}] = [SO_4^{2-}] = [S^0] = 0.01$ M,
$[Fe^{3+}] = [Fe^{2+}] = 0.015$ M.

In the light of the achieved voltages, it seems that something has prevented the full exploitation of the microbial culture's metabolic potential. The titanium wires and the carbon brush electrodes which produce minimal resistance were used in the MFCs. In addition, no leakages were observed during the experiment. If oxygen had been able to leak to the anode chambers, it could cause the drop of voltage, as the microbes would use oxygen rather than the anode electrode as the terminal electron acceptor. However, this should also mean that tetrathionate is still degraded which was not observed in the reactors faster than in abiotic control reactor. Thus, the most likely cause of poor performance in the reactors would be the limitation of mass transport because of insufficient mixing. Because there was no mixing, the biofilm may have lacked nutrients or substrate and have not therefore been able to produce electricity efficiently. As there was no significant electron flow, ferric iron reduction at the cathode remained low. Furthermore, the amount of biomass was too low for its accurate determination.

According to Valdés *et al.* (2008), *A. ferrooxidans* oxidises RISCs only in aerobic conditions but in anaerobic environments the organism may couple the oxidation of elemental sulphur to the reduction of Fe^{3+}. During the MFC experiment, yellowish white precipitate was accumulated to the bottom of the biotic reactors which could possibly be elemental sulphur granules. Precipitate was also observed in the abiotic control reactor which suggests that at least not all of it was elemental sulphur produced by the microorganisms but was perhaps some phosphate salt such as yellow $FePO_4$. Unfortunately, it was not possible to analyse the contents of the precipitate and thus its true composition remains uncertain. It could be that the accumulation of the elemental sulphur to the bottom of the reactors had deprived the cells from the material required for efficient metabolism which resulted in poor cell performance. This would, however, mean that the actual electricity producing process in the MFCs is not the one described in Equation 16, even though it describes best the full process of the tetrathionate degradation in the MFC, but rather the one described in Equation 13.

It has been suggested that in the S^0 oxidation of *A. ferrooxidans* the electrons are donated to *bc1* complex from which they enter the respiratory chain and are mediated via periplasmic transporters to Fe^{3+} in oxic and anoxic conditions (Corbett & Ingledew 1987). Another, more recent study suggests that H_2S may act as an intermediate in transferring the electrons to the ferric iron (Osorio et al. 2013). If the precipitate contained elemental sulphur, it could be that its loss led to the halting of the exoelectrogenic process and thus low electricity yields. The biomass concentration in the reactors was low, maybe as a result of poor energy conversion, which could also explain the low rate of tetrathionate degradation. Tetrathionate concentration most likely decreased in all MFCs due to the migration to the cathode compartment through the anion exchange membrane.

Furthermore, as the previous successful experiments with extreme acidophiles degrading tetrathionate to produce electricity (Sulonen et al. 2015) were conducted with a consortium of microbes from environmental sample, it is not clear if the two strains used in this experiment can power MFCs. Other possible organisms responsible of the actions in that particular MFC were *Acidihiobacillus thiooxidans* and *Ferroplasma acidarmanus* which were also detected in the study of Sulonen *et al*. (2015). The functionality of these organisms in MFCs should also be evaluated in more detail. *A. thiooxidans* is also capable of using reduced sulphur compounds to support growth (Kelly & Wood 2000) and *F. acidarmanus* is capable of chemolithotrophic growth with Fe^{2+} as the substrate (Baumler et al. 2005) and thus able to grow on substances that *A. thiooxidans* and *A. ferrooxidans* produce.

5.1.3 Future work

Three limiting factors were observed in the experiment that should be tackled when planning the future work. Firstly, as the lack of mixing may have caused the bad process performance, it would be feasible to repeat the experiments with adequate mixing.

Secondly, the inoculation of *F. acidiphilum* to the reactor should be ensured to be able to verify if the organism influences the performance of the MFC in negative or positive manner. The role of *F. acidiphilum* in the study of Sulonen *et al*. (2015) may be enhancing or impairing: It may enhance the performance of the MFC by e.g. providing mediators of electron transfer or by other exoelectronic activity. It may also impair the MFC performance by e.g. oxidising ferrous iron to ferric form and thus providing alternative terminal electron acceptors to *A. ferrooxidans*. Successful inoculation can be verified e.g. with PCR-DGGE analysis from the inoculation culture.

Lastly, another hindrance in the MFCs was the low amount biomass and thus methods to ensure the growth of more biomass should be evaluated. The aerobic cultures that degrade tetrathionate function by different mechanisms than those in anaerobic culture and thus inoculation of the MFCs with anaerobic cultures growing on tetrathionate and elemental sulphur with ferric iron as the electron acceptor could provide quicker starting culture. In

addition, different MFC start-up methods could be experimented. In this study, a stable 1000 Ω resistance was applied to the reactors which is not an optimal start-up method according to the literature presented in Section 2.3.3 nor the results discussed in Section 5.2.

5.2 MFC start-up strategies

The MFCs in the start-up experiment produced notably higher current densities than the MFCs in the tetrathionate degradation experiment. This may be due to the excellent substrate for the MFCs (sugars and acetate) as well as less hostile cultivation environment (near-neutral pH). The overall performance was promising and suggestions were made based on the obtained results.

5.2.1 The chemical oxygen demand of the system inclined in start-up

Soluble COD consumption was not observed in any of the MESs during the start-up phase of the experiment, but rather COD_s increased in the anolyte. The microbial consortia degraded the sugars fast from the anolyte but produced acetate instead of utilising it (Figure 4.14) which is one of the main factors of rising COD_s levels (Figure 4.13). The COD_s of the media did not match directly to the VFA concentrations towards the end of the experiment which means that the medium contained more compounds than detected in the analyses. The COD_s levels decreased in all reactors after they had been subjected to 47 Ω external resistance, and the GDR was able to consume not only sugars but also acetate from the media.

The increase of the soluble COD during the start-up phase was surprising and astonishingly large. The degradation of insoluble material to soluble substrates would be seen as the increase of soluble COD and it would provide the microbial culture nourishment thus eliminating the need of additional feeding. The COD_{tot} levels of the GDR were measured to see if the reactors contained high levels of non-soluble COD that could have been solubilised during the experiment. The COD_{tot} measurements from days 0 and 35 yielded 5.6 g/l and 5.4 g/l, respectively. The latter result is even lower than the corresponding COD_s result of the same day (6.1 g/l) which indicates that there may be some factors or substances that influence the measurement as the total COD should include all soluble COD. Additionally, it was analysed if the freezing of the sample has an effect on the measured COD and 14-day storage at -20 °C was found to lower the COD_s by 14.5% (measured from HAP sample on day 28) which was deemed as not sufficient decrease to explain the increasing COD.

Furthermore, it was analysed if the pre-treatment of the samples with different methods prior to COD analysis had an effect on the final result. This was due to a suspicion that

compounds such as iron, chloride, and hydrogen sulphide may affect the analysis (SFS 5504:1998). No significant differences compared to non-treated sample were found with utilisation of Hg^{2+} in the analysis (Cl^-), purging the sample with air (Fe^{2+}), nor with purging the sample with air through acidified sample (H_2S). Acidifying the sample and purging it with nitrogen (H_2S) increased the COD yield by 46% when the COD should have decreased after the pre-treatment if any disturbances were present. Thus, none of the tested potential disturbances account for the increase in COD.

The rising levels of soluble COD may also be caused by the unidentified compound found in abundance in the HPLC analyses. The inoculum may have contained high levels of polysaccharides that acted as the substrate after the utilisation of glucose and xylose. As the compound's observed retention time in the HPLC was close to but before those of other sugars, the compound might chemically resemble those sugars. However, according to HPLC the amount of compound did not seem to decrease substantially during the experiment but the dilutions of the samples were not optimal for the substance.

The inoculum originated from the anaerobic digester of a municipal wastewater treatment plant which is full of thick sludge from the wastewater treatment process. The first phase of anaerobic digestion is the bacterial hydrolysis where complex molecules such as polymers, polysaccharides, and fats are hydrolysed to smaller molecules (Evans et al. 2014). Therefore, the inoculum may have contained a high concentration of complex molecules as well as a microbial consortium that is capable of efficiently degrading it to soluble material. No significant difference was observed in the total and soluble CODs of the inoculum. However, the large amount of insoluble material hampered representative sampling from the inoculum and impeded the COD analysis in low dilutions with strong colour that prevented the titration of the sample.

The GDR provided the lowest overall COD_s levels and the second highest COD_s reduction (30%) when all the MFCs were subjected to same conditions. The GDR was also the only MES that was able to consume acetate the after total media change conducted on day 48. The HAP provided the highest COD_s reduction (40%) in 47 Ω R_{ext} but it also generated high amounts of COD after the total media change (+49%). Considering only soluble COD consumption, the most promising start-up method was to decrease external resistance from high to low gradually.

The initial COD_s in the wastewater was 2.5 g/l which resembles that of a real wastewater. Even higher COD loads have been utilised in previous studies, ranging from 1.0 to 18.6 g/l COD (Venkata Mohan et al. 2009; Pant et al. 2010). In the study of Venkata Mohan *et al.* (2009), the highest used substrate load (18.6 g/l COD) was still efficiently degraded. Though, Zhuang et al. (2012) reported that with loading rate of 1.2 g/l COD was degraded more efficiently in tubular MFCs than with loading rate of 4.9 g/l and Ge *et al*. (2014) claimed that the MFCs seem to perform the most efficiently under low substrate loads. The potential reasons for more efficient treatment with decreasing loading

rate were the lower accumulation of intermediates as well as the higher proportion of the electrogenic COD degradation (Zhuang et al. 2012). Even though the loading rate was not too high for the study, it might be feasible to examine how smaller loading rates would affect the MFC performance especially as the intermediates did accumulate to some extent in the present study.

5.2.2 High current was obtained from the high anode potential MFC

The maximal voltages attained from the MFCs during the control period with 47 Ω external resistance were 88.0–103.7 mV (Figure 4.11) which are significantly lower than what is achievable with the heterotrophic substrates (Table 5.2). The performance of the cathode may have restricted the electricity generation and thus the anode potential should be used as the measure of the anode performance. The lowest observed anode potentials during the control period were -472 mV to -491 mV in other MESs except LAP (-290 mV). The trend of electricity generation was inclining in the end of the control period and it would have been feasible to continue with the control period for longer time to be able to evaluate the performance of the reactors more precisely.

During the control period SLR and HAP showed increasing power production. These results are similar to those reported earlier by Lefebrve *et al*. (2011) for MFCs started with applying low external resistance and by various sources to MFCs started with controlled high anode potential (Finkelstein et al. 2006; Aelterman et al. 2006; Wagner et al. 2010; Kokko et al. 2015). However, it is peculiar that the SLR improved the COD consumption (Figure 4.13) and the electricity production during the control period, even though the external resistance (47 Ω) did not alter significantly from the initial resistance (50 Ω). The oscillating current curve produced by the MFC suggests that the decrease of the anolyte pH was affecting strongly and negatively to the overall performance of the reactor. The sensitivity of the MFCs to changes in the anolyte pH and composition is undesirable, considering that the applications would be treating industrial wastewater characteristics of which may alter with time.

Table 5.2 *The theoretical maximum cell voltages attainable from simulated forest industry wastewater used in this study.*

Reaction	E° (V)[a]	E vs. NHE (V)[b]	E vs. Ag/AgCl (V)[b]	E_{emf} vs. O_2 cathode (V)[c]
$CH_3COO^- + 4\ H_2O \rightarrow 2\ HCO_3^- + 9\ H^+ + 8\ e^-$	0.187	-0.278	-0.478	1.119
$C_5H_{10}O_5 + 10\ H_2O \rightarrow 5\ HCO_3^- + 25\ H^+ + 20\ e^-$	0.097	-0.422	-0.622	1.263
$C_6H_{12}O_6 + 12\ H_2O \rightarrow 6\ HCO_3^- + 30\ H^+ + 24\ e^-$	0.105	-0.417	-0.617	1.258
$O_2 + 4\ H^+ + 4\ e^- \rightarrow 2\ H_2O$	1.229	0.841	0.641	0

[a] Calculated with Equation 5, [b] Equation 8, [c] Equation 9

The conditions used in the calculations: $[HCO_3^-]$ = 1 g/l, [acetate] = 1. g/l, [glucose] = 0.66 g/l, [xylose] = 0.33 g/l, pH =6.5, $p(O_2)$ = 0.21 atm, T_{anode} = 37 °C, $T_{cathode}$ = 20 °C.

Considering the electrochemical performance as well as the COD consumption, the most successful start-up strategy was by controlled high anode potential. The HAP produced current throughout the study and outperformed the other MESs in voltage production on many occasions but did not consume COD_s before the control period of the study. It is yet promising that the COD utilisation was high during the control period when also the voltage production elevated steadily.

LAP performed poorly throughout the study: it produced the most COD and generated the least current. In many studies the poised low anode potential has been found the least desirable start-up method for MFCs (Wagner et al. 2010; Kokko et al. 2015), even though also contradictory studies exist (Torres et al. 2009; Wagner et al. 2010). According to Wagner *et al*. (2010), there are notable exceptions among the exoelectronic microorganisms which require more negative anode potentials. They also suggest that the potential of the terminal respiratory proteins is more important to the growth conditions than the potential of the anode electrode and that more negative anode potentials may lead to higher current densities in functioning MFCs (Wagner et al. 2010). It may be that the applied anode potential (-450 mV vs. Ag/AgCl) was too low for the organisms and thus limited the biofilm formation at the anode. As the other three reactors were able to produce anodic potentials lower than -450 mV, it cannot be concluded that the anode potential would have been lower than that of the respiratory proteins of the organisms.

5.2.3 Future work

Differences were observed between the MFC start-up methods in the experiment. However, some suggestions can be made regarding future studies. As the high controlled anode potential and the low external resistance were deemed the most successful strategies in this setting, the natural continuum would be to study the optimal high anode potential and optimal low external resistance for the start-up.

The start-up period of the MFCs was rather long due to the interval at which the external load of one reactor was adjusted. The anodic potential of the reactor remained low (under -400 mV) until the external resistance was decreased to 100 Ω (Figure 4.9) which indicates that the external resistance could have been decreased faster. The start-up period could thus be shortened significantly, and the control period extended likewise, to allow the emergence of even clearer differences between the MFCs started using different strategies.

The unidentified compound found in the anolyte should be identified to make sure what the reactors were utilising for the electricity generation. This would also allow more precise determination of the Coulombic efficiency. Lower starting COD could benefit the study by minimising the accumulation of intermediates to the medium.

6. CONCLUSIONS

MFCs are an important future technology with possible applications in many industrial sectors, such as wastewater treatment and mining industry. Wastewaters are huge polluters of the environment globally, especially in the developing countries, that lack efficient governmental control of discharges. Industrial wastewaters are typically concentrated and they contain chemical energy which is lost in the wastewater treatment procedures if traditional methods such as chemical precipitation or activated sludge process are used. MFC technology is a step forward to circulation economy where even the waste is utilised to its full potential, as it allows the amalgamation of wastewater treatment and electricity generation from the chemical energy present in the wastewater. But for now, MFC technology is in its infancy and rigorous research is required for more viable industrial applications.

The aerobic batch bottle experiments conducted with pure cultures of *A. ferrooxidans* and *F. acidiphilum* suggest that the former is the effective tetrathionate degrading organism. However, no current generation was achieved in the MFCs inoculated with *A. ferrooxidans*, most likely due to insufficient biomass generation which resulted from mass transfer limitations caused by inadequate mixing. The exact amount of biomass was not, however, determined. More research is needed to verify the role of *A. ferrooxidans* as an exoelectrogen.

Based on electricity generation, controlled high anode potential was found to be the most recommendable start-up strategy for the MFCs. Applying low external resistance is also a promising option if expensive potentiostats are not available. None of the studied methods could consume COD constantly from the medium. An unidentified compound was detected in HPLC analysis which may have caused the increase in COD levels during the experiments. Gradually decreasing external resistance provided the highest COD_s degradation efficiency but its current generation was low and as a start-up method it is slower than the other methods it was compared to. However, the start-up times can be shortened significantly with fewer of steps and shorter times with each resistor. Following experiments should aim to shorter start-up periods and more stable decrease of COD.

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